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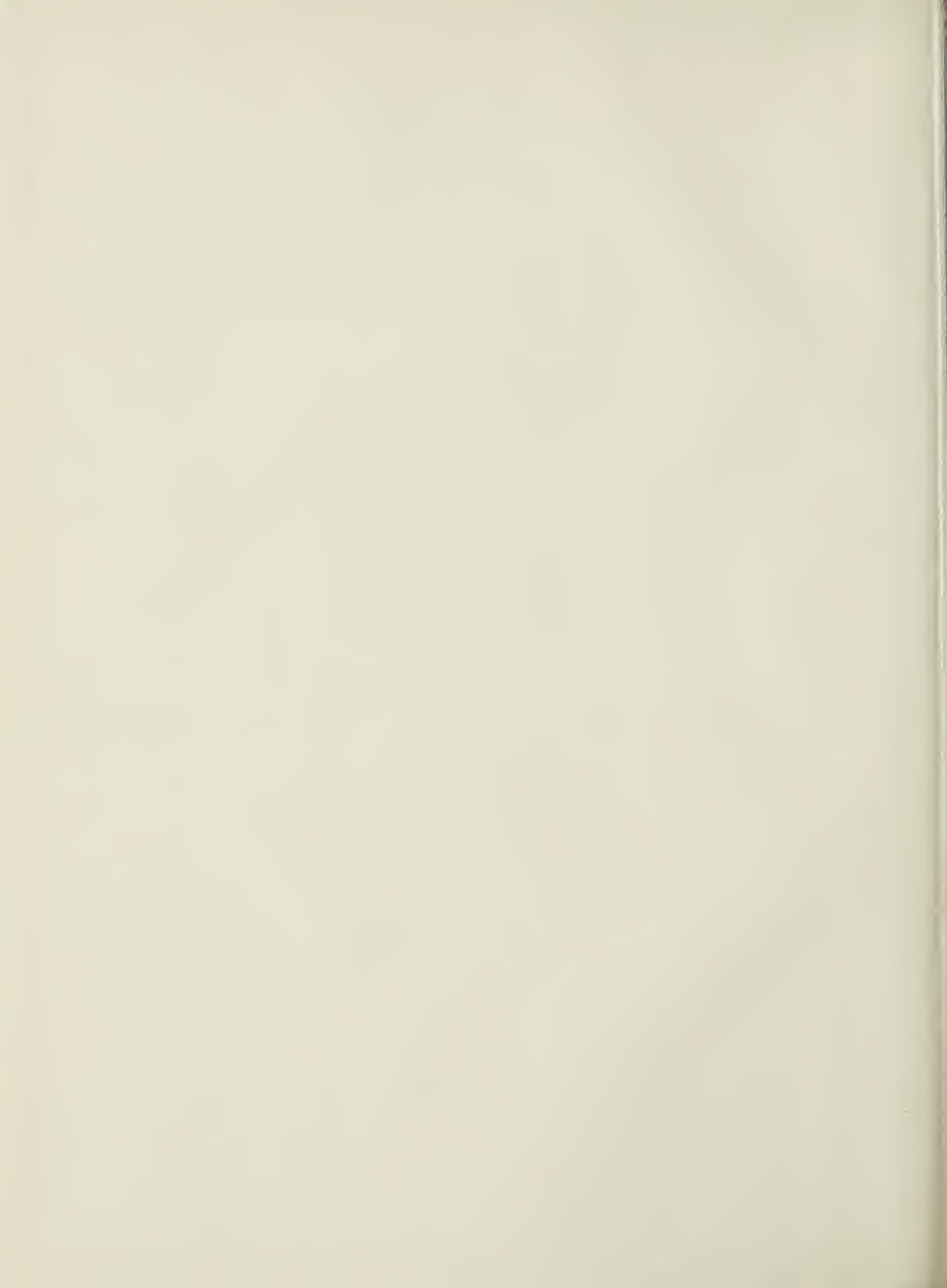
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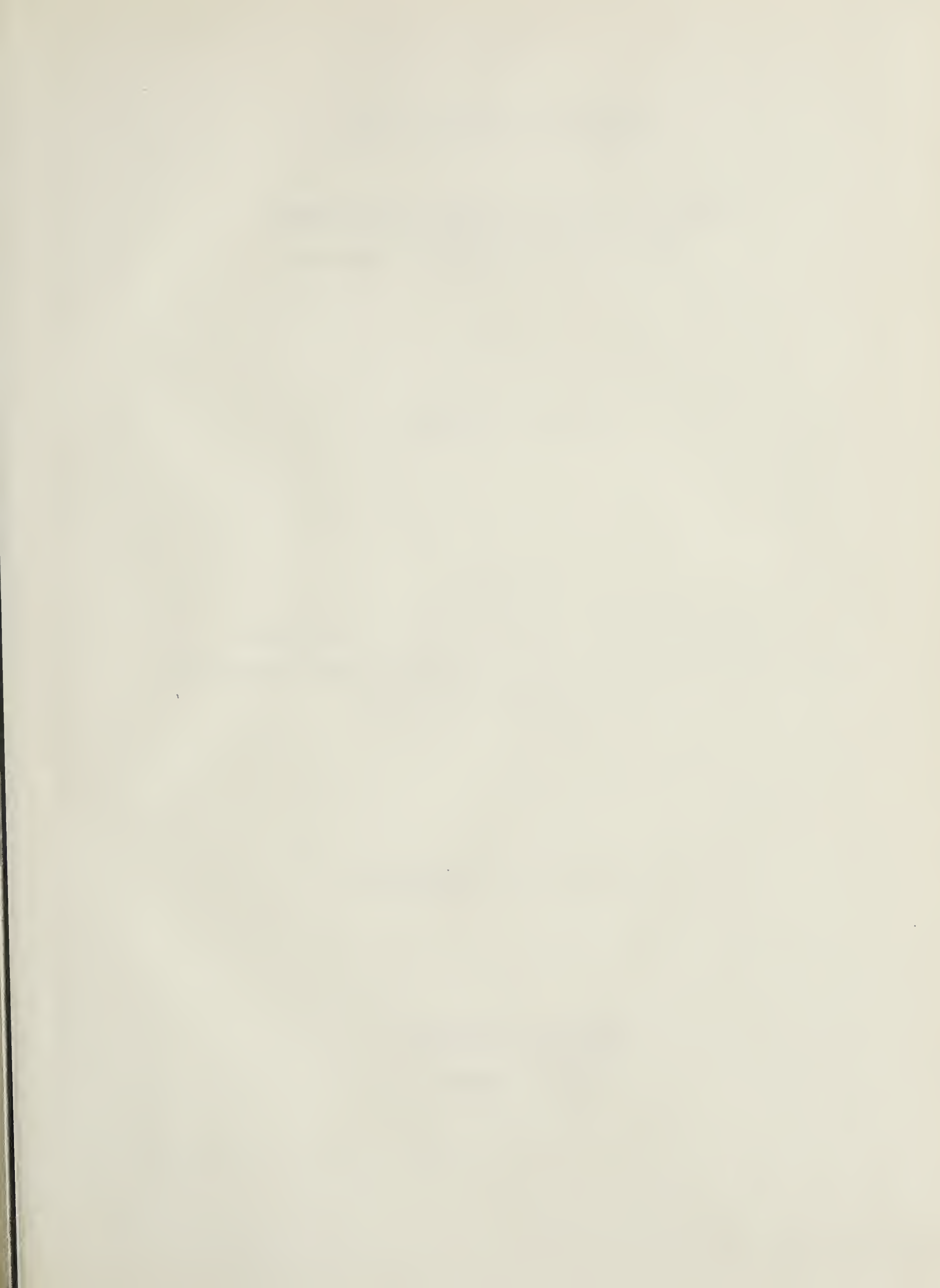


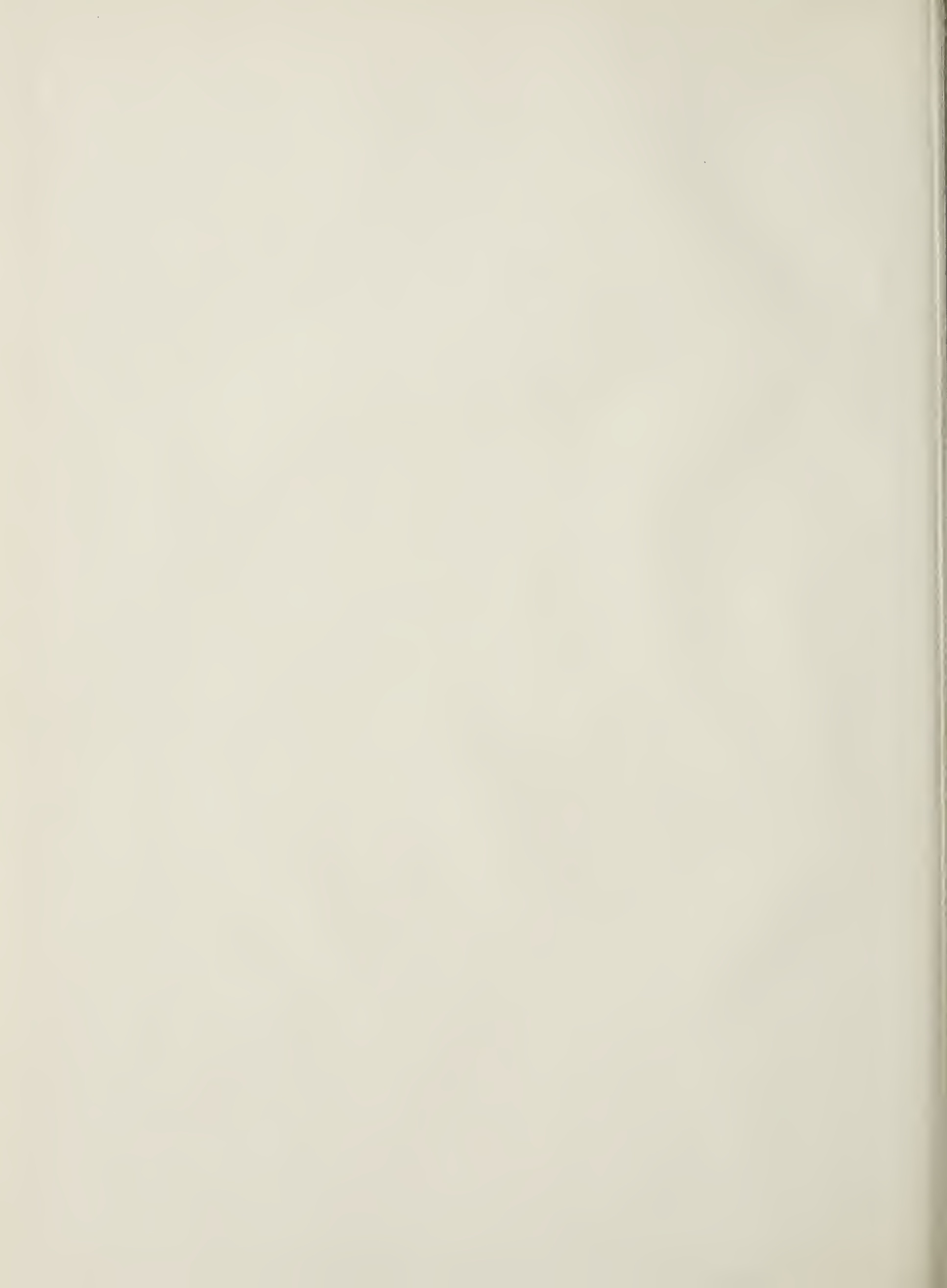


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COMPARATIVE STUDIES OF THREE VARIANTS
OF MENGO ENCEPHALOMYELITIS VIRUS

by

JAMES B. CAMPBELL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "COMPARATIVE STUDIES OF THREE VARIANTS OF MENGO ENCEPHALOMYELITIS VIRUS", submitted by James B. Campbell in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

The biological properties of three variants of Mengo encephalomyelitis virus have been studied. The variants, serologically very closely related, were originally isolated on the basis of the size and morphology of the plaques they produce in monolayers of Earle's L strain of mouse fibroblasts under agar overlay. They were designated S-Mengo (sharply defined, minute plaques), M-Mengo (sharply defined, medium-sized plaques), and L-Mengo (large, diffuse plaques).

It has been shown that the differences in the size of the plaques produced by the three variants can be explained on the basis of their differing sensitivities to sulfated agar components. Agar appears to contain at least two fractions inhibiting S- and M-Mengo plaque development, only one of which can be extracted with saline. In a suspended cell system, the interaction between L cells and S-, M-, and L-Mengo is inhibited to the extent of 50% by the saline-extracted material (agar factor) at concentrations of 0.4, 3.3, and 35 $\mu\text{g/ml}$, respectively.

A preliminary physical and chemical analysis of the agar factor indicates that it is a homogeneous sulfated polysaccharide (3% sulfate) of molecular weight 50,000, containing galactose and probably 3,6-anhydrogalactose and pyruvic acid.

Protamine, which when added to agar overlay reverses the inhibition of plaque formation imposed by the agar inhibitors, is itself a potent inhibitor of cell-virus

interaction in the suspended cell system. The S variant is most sensitive to inhibition by this polycation, while L- and M-Mengo exhibit sensitivities of the same order of magnitude.

Data are presented which indicate that the agar factor blocks cell-virus interaction by directly immobilizing the virus particles, while protamine acts by blocking receptor sites on the cell surface.

The rates at which the three variants attach to L cells, in monolayers and in suspended culture, have been determined. In both systems, the L variant attaches much less rapidly than do the other two variants. In the monolayer system, the M and S variants attach to L cells at much the same rate, while in the suspended cell system, the S variant appears to attach more readily than does the M variant.

pH has a dramatic effect on the infectivities of the M and S variants for L cells, in both monolayer and suspended cultures. Increasing the pH at which cells and virus interact from 6.8 to 7.8-8.0, increases the apparent titer (as determined by plaque assay) of the L, S, and M variants by factors of 2, 10, and 3000, respectively, although over this range, no effect of pH was noted on the infectivity titers of six poliovirus strains. The effect of pH on the Mengo variants has been shown to be due, in part, to the instability of the M and S variants at pH's less than 7.2; an instability apparently dependent upon the presence of chloride ions in the suspending medium.

Detailed single cycle growth curves of the variants in suspended L cell cultures at 37° have revealed that L-, S-,

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and M-Mengo have burst sizes of about 200, 400, and 1000 PFU/cell, respectively. Mature particles of M-Mengo are produced after an eclipse period of about 4 hours; those of S- and L-Mengo, however, are not formed until 5.5-6 hours after initial infection. With all three variants, virus is first released from the cells 1.5-2.5 hours after its intracellular appearance, although even at 12 hours post-infection, only 20-40% of the total virus is extracellular.

The distribution of infectious virus particles in various tissues of the mouse has been determined following the intraperitoneal injection of the variants. When lethal doses are administered, virus appears first in the spleen and lymph nodes, followed by its appearance in the spinal cord and brain, in which tissues the infectious titers increase progressively until the death of the animals. Low levels of virus are sometimes found in the lung, kidney, and heart at 24-48 hours post-challenge, but they subsequently disappear from these tissues. Virus has never been detected in the blood and liver of infected 24-26 g mice, although low levels have been recovered from the blood of 14-16 g mice. When sublethal doses are injected, virus is detectable only in the spleen and lymph nodes, and disappears from these tissues by 8-9 days post-challenge.

The LD₅₀'s of the three variants when injected by both the intraperitoneal and intracerebral routes were determined. The intraperitoneal LD₅₀'s for the L, M, and S variants in 14-16 g mice were found to be 1, $1-5 \times 10^4$, and probably $>10^7$ PFU, respectively. The intracerebral LD₅₀'s are essentially

the same (1-5 PFU) for all three variants.

The L variant is adsorbed to mouse and chicken tissue homogenates to a considerably lesser extent than are the other two variants. With the possible exception of a reduced affinity of S-Mengo for brain tissue, there is no significant difference in the extent to which the M and S variants are adsorbed to homogenates of mouse brain, spleen, liver, heart, and kidney.

Of the three variants, the growth of L-Mengo appears to be the most strongly inhibited by the presence of exogenous, homologous interferon. Indications have also been obtained that intraperitoneal injection of the L variant in mice elicits the production of a considerably larger amount of interferon than does the intraperitoneal injection of a similar infectious dose of S-Mengo.

The effect of a number of different overlays on the sizes of plaques produced by the Mengo variants has been examined, and the respective merits of agarose, agar containing protamine, and methylcellulose as inhibitor-free overlays have been compared.

The addition of several anionic polymers (agar factor, dextran sulfate, heparin, chondroitin sulfate, polyphloroglucinol phosphate, and polyphloreitin phosphate) to regular agar overlay has been shown to have little or no effect on the sizes of plaques produced in L cell monolayers by L- and S-Mengo. M-Mengo plaque size, however, is greatly affected: it may be either decreased or increased, or both, by individual polyanions. Data are presented suggesting that molecular

weight is an important factor in determining the ability of a sulfated polysaccharide to enhance M-Mengo plaque size: lower molecular weight preparations of dextran sulfate and sulfated hyaluronate enhance plaque size more efficiently than do higher molecular weight ones containing the same amount of sulfate. Efforts to explain the mechanism of the plaque enhancement by low molecular weight polyanions have not been successful. It has been shown, however, that the enhancement of plaque size is not brought about by an increase in burst size or by an earlier release of virus from infected cells.

L-Mengo is markedly sensitive to heparin, and the M and S variants are relatively insensitive. In a suspended cell system, the interaction between L cells and L-, S-, and M-Mengo is inhibited to the extent of 50% by heparin at concentrations of 2, 50, and 110 $\mu\text{g/ml}$, respectively.

The isolation of six distinct new variants from pools of S- and M-Mengo is described. The plaque sizes of five of the six are strongly inhibited by polyanions. The sixth, although similar to L-Mengo in its virulence to mice and in that its plaques are almost unaffected by the addition of protamine or dextran sulfate to the agar overlay, can be distinguished from the latter by the larger and more uniform plaques it produces. The intraperitoneal LD_{50} 's of all six new variants in 14-16 g mice have been determined.

The significance of the results of the above studies are discussed, and an attempt at synthesis is made.

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ABBREVIATIONS

EMC	- encephalomyocarditis
VSV	- vesicular stomatitis virus
RNA	- ribonucleic acid
BPA	- bovine plasma albumin
PBS	- phosphate buffered saline
TBS	- TRIS buffered saline
TRIS	- tris(hydroxymethyl)-aminomethane
DEAE	- diethylaminoethyl
PFU	- plaque forming unit
LD ₅₀	- 50% lethal dose
k	- initial rate constant
S _{20,w}	- sedimentation coefficient in water at 20°

All temperatures are given in Centigrade degrees.

INTRODUCTION

In 1946, Dick and coworkers isolated an apparently hitherto unknown virus from the brain and cerebrospinal fluid of a paralysed captive rhesus monkey. The agent was subsequently isolated by these workers from mosquitos, a mongoose, and from man (Dick himself). It was named Mengo encephalomyelitis virus because it was isolated in the Mengo district of Buganda, Uganda, and because it produced lesions in the brain and spinal cord of infected animals and encephalitic symptoms in man (Dick et al., 1948a,b; Dick, 1948).

Successive brain to brain passages rendered the Mengo virus extremely lethal to mice when administered intracerebrally, intraperitoneally, or intranasally (Dick, 1948). Subsequent studies showed that it had no antigenic relation to the Lansing, Yale-SK, or MEF-1 strains of rodent-adapted poliomyelitis virus, or to any of the Theiler's mouse encephalitis viruses, but was serologically identical to the Columbia-SK (Jungeblut and Sanders, 1940), MM (Jungeblut and Dalldorf, 1943), and encephalomyocarditis (Helwig and Schmidt, 1945) strains of virus (Dick, 1949; Warren et al., 1949a). More recent studies, however, have shown that, although closely related, these viruses are not completely identical (Ellem and Colter, 1961; Craighead, 1965).

The present thesis is mainly concerned with the biological properties of Mengo encephalomyelitis virus. Nevertheless, in view of the close antigenic relationship between it and the

Columbia-SK, MM, and encephalomyocarditis (EMC) strains, the following discussion will not be confined to a description of Mengo alone, but will deal with properties of all these viruses, collectively known as the Columbia-SK group.

Physico-chemical studies of Columbia-SK viruses have shown that they are probably composed exclusively of RNA and protein (Faulkner et al., 1961; Hausen and Schäfer, 1962), and are presently classified as picornaviruses (Andrewes, 1964). Ribonucleic acid is probably the genetic material, as evidenced by the numerous reports of isolation of infectious RNA from viruses of the group, e.g. Mengo virus (Colter et al., 1957), EMC virus (Bellett and Burness, 1963), and mouse encephalitis (ME) virus (Franklin et al., 1959). This last-named virus has no antigenic relation to the Theiler's mouse encephalitis viruses, and has been shown to be a member of the Columbia-SK group (Hausen and Schäfer, 1962). It has also been called Maus-Elberfeld virus (Rueckert and Schäfer, 1965).

Faulkner et al. (1961) analysed purified, crystalline preparations of EMC virus, and obtained a value of 1×10^7 for the particle weight. The preparations contained around 30% RNA, giving a value of 3×10^6 as the weight of the RNA, assuming one molecule of RNA per virus particle (Faulkner et al., 1961). Hausen and Schäfer (1962) obtained a particle weight of 5.7×10^6 for crystalline ME virus, and calculated a molecular weight of 2×10^6 for its RNA. As estimated from electron micrographs, the particle size of ME virus was apparently also slightly smaller than that of EMC: 24 m μ diameter as opposed to 27 m μ (Hausen and Schäfer, 1962; Faulkner et al.,

1961). Dales and Franklin (1962) have found the structure of Mengo and EMC virus to be identical and to have particle diameters of 27-28 m μ , although Hinz et al. (1962) have estimated a diameter of 20 m μ for EMC particles, and measurements in this laboratory have indicated that Mengo virus has a diameter of 21-22 m μ .

It seems unlikely that these differences are real, and are probably due either to differences in technique or to ways of measurement. Of more import is the fact that published electron micrographs of Columbia-SK viruses, and electron micrographs of the Mengo virus preparations used in the present study, all indicate a very similar or identical structure. In each case, the spherical protein envelope of the virus particles consists of subunits, probably with a diameter of about 4-5 m μ , and it is likely that the Columbia-SK viruses belong to the large group of small, spherical viruses, including poliovirus, showing icosahedral symmetry of their surface subunits (see e.g. Klug and Caspar, 1960).

The structural protein components of ME, EMC, and Mengo viruses contain two major electrophoretic components of weight-average molecular weight about 26,000, which have been shown to exist in the virion unlinked to each other by disulfide bridges (Rueckert, 1965). These components are remarkably similar in electrophoretic behavior to the two major structural components of poliovirus isolated by Maizel (1963, 1964). It is interesting to note also that Hamilton et al. (1962) have reported the isolation from EMC virus of an antigenically active subunit of molecular weight about 60,000, which could

possibly be a composite of two or three of the subunits isolated by Rueckert.

Analyses of the amino acid composition of whole virus have revealed that the protein of EMC, ME, and poliomyelitis virus are closely similar (Faulkner et al., 1961; Rueckert and Schäfer, 1965; Munyon and Salzman, 1962). The most striking difference appears to be in the comparatively low cysteine content of poliovirus, although even this may be artifactual (Rueckert and Schäfer, 1965). It is apparent that the similarity between the viruses of the Columbia-SK group and poliomyelitis extends beyond the morphologic to the structural level.

In the 25 years since the isolation of the first member, viruses of the Columbia-SK group, or neutralizing antibodies to them, have been recovered from a wide range of species on four continents. These include wild rats (Warren et al., 1949b), voles and squirrels (Vizoso and Hay, 1964), and pigs (Murnane et al., 1960). Antibody to encephalomyocarditis virus has also been obtained from convalescent sera from U. S. army troops in Manila suffering from an outbreak of "three day fever" (Smadel and Warren, 1947). Although at least one member (Mengo) has been isolated from mosquitos (Dick et al., 1948a), insects do not appear to be an important vector in the transmission of viruses of the group. The actual means of transmission remains unknown, although nasal absorption has been suggested as a possible route of infection of rodents (Vizoso and Hay, 1964).

The distribution of Columbia-SK viruses in animals following injection appears to vary with the strain and host employed. Forty-eight hours after the intraperitoneal injection

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of adult mice with the original Mengo strain, virus was recovered in high titer from the brain and spinal cord, and only in low titer from spleen, kidney, liver, heart, and lung (Dick, 1949). A pig which died after the oral administration of EMC virus contained large amounts of virus in the heart, and relatively large amounts in the spleen and mesenteric lymph nodes (Craighead et al., 1963), indicating that lymphatic tissue was a site of virus propagation. The brain was apparently not examined.

Route of administration can alter the pathogenic properties of the agents: Jungeblut and Steenberg (1950), for example, have shown that intracerebral passage modifies the cardiotropic properties of Columbia-SK and EMC virus in mice. By passaging in different ways, Columbia-SK virus has been adapted to cause predominantly lesions of the nervous system or of muscles (Kuwata, 1956). A variant of Columbia-SK virus has been described which is lacking in neurovirulence (Furusawa and Cutting, 1962) and Craighead (1965b) has selected an EMC variant which causes a non-fatal infection in mice manifested primarily by myocarditis.

In cultured cells, viruses of the Columbia-SK group produce very similar morphological alterations. In a study of the growth of EMC virus in embryonic mouse cells, Hinz et al. (1962) observed that nuclear alterations appeared prior to significant cytoplasmic changes and before virus particles were visible. These alterations included margination of the chromatin, the appearance of nucleolar condensations and of a dense granular substance in the interchromatinic zone, followed

by nuclear shrinkage. Dales and Franklin (1962) also noted nuclear alteration in mouse L fibroblasts infected with both Mengo and EMC viruses.

A considerable increase in cytoplasmic basophilia, connected with the increase of free ribonucleoprotein particles, has been observed in Mengo and Columbia-SK infected cells (Barski, 1957). At an early stage in the infection with EMC virus, Ginsburg-Tietz et al. (1964) found a transfer of ribonucleoprotein particles from the nucleus to the cytoplasm of Krebs-2 tumor cells. These observations appear to be related to the study of Martin and Work (1961) who showed that within 4 hours after infection of Krebs-2 cells with EMC virus, a transfer of RNA took place from the nucleus to the cytoplasm. That this was viral RNA is doubtful, however, from the more recent work of Dalgarno and Martin (1965) who, using the same system, concluded that viral RNA is synthesized in the large-particle fraction of the cytoplasm. Franklin (1962) found no evidence for the multiplication of either Mengo virus RNA or protein in the nucleus of L cells, but found viral antigen first in a perinuclear location and later in cytoplasmic inclusions. This is in contrast to the work of Bellett and Burness (1963) and Zalta et al. (1963), both of which groups obtained infectious viral RNA from nuclear fractions of Krebs-2 ascites cells infected with EMC virus (see also Sanders, 1962). The observation that the viral genome of Mengo is specifically localized in polyribosomes from the earliest stages of infection onward, remaining undegraded (Tobey, 1964a,b) suggests that the synthesis of viral RNA is

cytoplasmic rather than nuclear, but this point still remains to be clarified (Bellett et al., 1965). The changes taking place in the cell nucleus following viral infection do not in themselves signify that viral synthesis is occurring there, since they may simply be consequences of the shut-down of normal cellular RNA and protein synthesis which results soon after initiation of viral infection (see Franklin and Baltimore, 1963).

Several methods of assaying the infectivity of Columbia-SK viruses exist. The original one, by intracerebral or intraperitoneal injection of mice, is still in use (see e.g. Speir et al., 1962) although the limitations of this method are obvious. A more convenient one takes advantage of the fact that many strains of the viruses of this group hemagglutinate sheep red cells (Jungeblut, 1958). Sanders (1957) has used a cell-death titration to estimate EMC virus in Krebs-2 ascites cells--a process involving counting the percentage of dead cells by staining with nigrosin 15 hours after infection with virus. Although Krebs-2 cells will not grow as a monolayer on a solid substrate, Sanders (1957) has developed a plaque assay for EMC virus in these cells by infecting with virus and suspending them in warm agar. Another plaque assay, and probably the one in most common use now, takes advantage of the fact that Columbia-SK viruses grow well in Earle's L strain of mouse fibroblasts--a continuous cell line which is easily cultivated in suspension or on a glass or plastic substrate. Monolayers of L cells are readily prepared, and Mengo and EMC viruses have been found to produce clearly-defined



S-MENGO

M-MENGO

L-MENGO

Figure A.1. Plaques produced by the three variants of Mengo encephalomyelitis virus in monolayers of L-929 mouse fibroblasts under nutrient agar overlay. The plaques were visualized by staining with neutral red after 72 hours' incubation at 37° in an atmosphere of 5% CO₂ in air.

plaques in them (Ellem and Colter, 1960; Takemoto and Liebhaver, 1961; etc.).

During the course of a study of the interaction of infectious Mengo RNA with L cells (see Colter and Ellem, 1961), it was noticed that the plaques produced in L cell monolayers infected with the viral RNA were markedly heterogeneous in size. A similar heterogeneity was observed when monolayers were infected with virus from the pools used for the isolation of the infectious RNA. By selecting several of the largest and smallest plaques for cloning, two distinct variants were isolated: a small plaque variant yielding only minute plaques after three days' incubation, and a large plaque former. The latter yielded plaques with a considerable variation in size, but repeated cloning did not reduce this heterogeneity. By passaging the large plaque former in monkey kidney cells, a third variant was isolated, which produced plaques of diameter intermediate between those of the small and large plaque formers. Pools were prepared of these variants, which were designated S-, M-, and L-Mengo, for the small, medium, and large sized plaques they produced in L cell monolayers (Ellem and Colter, 1961). Figure A.1 shows their characteristic plaque morphology.

The simplicity of detection of plaque-type variants has resulted in their isolation from numerous types of virus. In many cases, variants which fall into this category are either resistant or sensitive to sulfated polysaccharide inhibitors in agar (Liebhaver and Takemoto, 1963), and it is this property which determines their plaque morphology under agar overlay.

With other viruses, however, this is not the reason, and other explanations for the occurrence of large and small plaque variants have been advanced. The small plaques produced by some variants of poliovirus, for example, have been shown to be due probably to their reduced burst size (Carp and Koprowski, 1962), and large plaque variants of adenovirus type 5 and of vesicular exanthema of swine (Kjellén, 1963; McClain and Hackett, 1959) appear to be released from their host cells more rapidly than the corresponding small plaque formers. A variant of foot and mouth disease virus producing large plaques was more acid-stable (Mussgay, 1959), and with a large and a small plaque variant of vesicular stomatitis virus, the latter was found to be the more sensitive to inhibition by exogenous interferon (Wagner et al., 1963).

In the report describing the isolation of the three variants of Mengo virus, Ellem and Colter (1961) concluded that the marked differences in the size of the plaques produced by the variants could not be explained on the basis of inhibition by an agar component, although they did not offer an alternative explanation. Since then, a detailed study of S-, M-, and L-Mengo has been in progress, the objective being: (i) to uncover the mechanism underlying the differences in the size and morphology of the plaques they produce, (ii) to define, as precisely as possible, a broad spectrum of their biological characteristics, and (iii) to relate these biological markers to the biochemical and biophysical parameters of the virus particles. The following work summarizes data pertaining to the first two parts of this objective.

ROUTINE MATERIALS AND METHODS

Mengo encephalomyelitis virus

The virus was obtained originally from Dr. Kenneth Smithburn of the Division of Medicine and Health of the Rockefeller Foundation. It had been maintained by periodic intracerebral passage in 10-12 g Swiss mice, and the pools from which the variants were isolated were prepared by propagating the virus in Ehrlich ascites tumor cells in vivo (Ellem and Colter, 1961).

Tissue culture media

All media were made up in distilled, deionized water.

Growth medium. Earle's balanced salt solution (Earle, 1943) with ten times the usual concentration of Na_2HPO_4 , containing double the concentration of vitamins and amino acids recommended by Eagle (1955) and either 10% horse serum or 10% calf serum for the growth of L cells or HeLa cells respectively. The vitamins, amino acids, horse serum and calf serum were obtained from Baltimore Biological Laboratory, Baltimore, Md.

Spinner medium. Identical to the above, except that calcium was omitted.

Virus diluent. The buffered balanced salt solution (PBS) described by Dulbecco and Vogt (1954) to which bovine plasma albumin, fraction 5 (Pentex Inc., Kankakee, Ill.) was added to a final concentration of 0.2%.

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Overlay diluent. A solution of three-times strength Hanks' balanced salt solution (Hanks and Wallace, 1949) containing 30% heat-inactivated calf serum, 0.75% sodium bicarbonate, and six times the usual quantity of Eagle's nutrients. The calf serum was inactivated by heating at 56° for 30-60 minutes with occasional shaking.

Regular agar overlay. Two volumes of 1.5% Noble agar (Difco Laboratories, Detroit, Mich.) mixed with 1 volume of overlay diluent. Of this, 4.5 ml was used routinely to overlay cell monolayers.

Phenol red. Phenol red (Fisher Scientific Co., Fair Lawn, N. J.) was incorporated in all media as a pH indicator, at a final concentration of 0.02%.

Antibiotics. Sodium penicillin G (Ayerst, McKenna and Harrison, Montreal, Que.) and streptomycin sulfate (Strepolin '33'; Glaxo-Allenburys Ltd., Weston, Ont.) were incorporated in all media at final concentrations of 100 I.U./ml and 100 µg/ml respectively.

Maintenance of cells

The continuous cell lines used in this study--the L-929 strain of mouse fibroblasts, and a strain of HeLa cells--were grown in suspension at 37° in spinner medium, in large flasks fitted with side arms and a magnetic stirring device (Bellco Biological Glassware, Vineland, N. J.). Fresh suspended cell cultures were prepared weekly by trypsinization of monolayer cultures propagated in growth medium in Pavitsky bottles. The trypsinization procedure consisted of incubation of the cell monolayers with 0.25% trypsin in Hanks' balanced salt

solution until the cells detached from the glass, whereupon the cells were spun down, washed, and resuspended in fresh spinner medium.

Preparation of cell monolayers

Two and a half million cells in 6 ml of growth medium were introduced into 60 mm plastic petri dishes (Falcon Plastics, Los Angeles, Calif.). After incubation at 37° for 24 hours in a humidified atmosphere of 5% CO₂ in air, confluent monolayers were obtained containing about 6×10^6 cells, and were then ready for use.

Preparation of virus pools

Pools of Mengo virus were prepared by infecting suspended cultures of L-929 cells with virus at high multiplicity, and harvesting the fluid after lysis 18-24 hours later or when the viable cell count was less than 10% of the original. Pools of poliovirus were prepared in HeLa cells in the same manner. As reported by Fuerst (1961) with EMC virus, heating the L cells for 1 hour at 42° prior to infecting them with Mengo virus resulted in the production of virus pools of considerably increased titer, and this modification was utilized in some preparations. The virus pools were stored in small aliquots at -70°.

Plaque assay

This was the standard method of assaying virus preparations. Small volumes, usually 0.1 ml, of appropriate dilutions of the virus samples in virus diluent, were pipetted onto pre-formed monolayers of susceptible cells. After incubation for one hour at 37°, during which time the virus particles attached

to cells in the monolayers, 4.5 ml of regular agar overlay was applied to each. After 2-3 days incubation at 37° in an atmosphere of 5% CO₂ in air, the plates were overlaid with another 3 ml of regular agar overlay, this time containing neutral red (Fisher Scientific Co., Fair Lawn, N. J.) at a concentration of 1:10,000 (w/v). Virus plaques were scored when visible several hours later. With all the Mengo variants, plaque counts were near maximal on the third day.

Sterilization procedures

The balanced salt solutions, and all media containing protein or bicarbonate were sterilized by filtration through a Seitz filter, the first 50-1000 ml of each filtrate being discarded, depending on the size of the filter. Agar suspensions were sterilized by autoclaving at 125° for 15 minutes. Neutral red solutions were also autoclaved, but were found to be less toxic to L cells when sterilized by filtration. This has been previously noted by Wallis et al. (1962) and Nagai and Hammon (1964). All media were checked for sterility by incubating samples in sterile thioglycollate medium (Baltimore Biological Laboratory, Baltimore, Md.) and sterile brain heart infusion medium (Difco Laboratories, Detroit, Mich.).

Glassware which had been in contact with virus was decontaminated by overnight immersion in Wescodyne solution (West Chemical Products, Inc., Montreal, Que.) followed by rinsing, washing with detergent, further rinsing, and sterilization in an oven at 375° for 2.5 hours. Pipettes were immersed in Wescodyne solution for 24 hours, rinsed, transferred to concentrated H₂SO₄ for another 24 hours, thoroughly

rinsed in tap and distilled water, and sterilized in an oven in the same manner. Although Wallis et al. (1963) have found Wescodyne to be unsatisfactory as a virucidal agent, especially for poliovirus and other enteroviruses, it seems highly unlikely that any virus remaining active after treatment with this agent would survive the rest of the cleaning and sterilizing procedures.

1. The first part of the paper discusses the importance of the study and the objectives of the research. It also provides a brief overview of the methodology used in the study.

2. The second part of the paper presents the results of the study. It includes a detailed analysis of the data collected and a discussion of the findings.

3. The third part of the paper discusses the implications of the study and provides recommendations for future research. It also includes a conclusion and a list of references.

4. The fourth part of the paper is a summary of the study and its findings. It provides a brief overview of the main points discussed in the paper.

5. The fifth part of the paper is a list of references. It includes a list of all the sources used in the study.

CHAPTER 1

Growth of the Mengo Variants in L Cells

Introduction

Pools of M-Mengo prepared in L cells almost invariably have higher titers than similarly prepared pools of S-Mengo, and usually contain 5-10 times as much infectious virus as do those of the L variant. The relatively low titer of L-Mengo pools is surprising, since of the three variants, L-Mengo produces by far the largest plaques under regular agar overlay.

The purpose of the present chapter is to record attempts to reconcile these apparently anomalous observations. Although it seemed unlikely that L-Mengo could produce more infectious progeny virus per cell (i.e. have a higher burst size) than the other two variants, the possibility remained that with this variant, progeny virus was released earlier from infected cells, thereby spreading more rapidly in L cell monolayers and producing the largest plaques. The growth characteristics of the three variants in suspended L cells over a period of 24 hours were therefore determined, and the results of these studies are presented here.

Materials and Methods

Virus pools

In order to infect a major percentage of the L cells in suspension, it was necessary to use an input virus multiplicity of 100 with each variant. This was especially necessary

THE HISTORY OF THE

REPUBLIC OF THE UNITED STATES

The history of the Republic of the United States is a story of growth and development. It begins with the first settlers who came to the shores of the New World, seeking a better life and a new land. They found a land of vast resources and a people who were different from them. The settlers and the natives lived together for many years, but the relationship was not always peaceful. The settlers wanted to expand their territory and the natives wanted to protect their land. This led to a series of wars and conflicts. The settlers won the wars and the natives were driven from their land. The settlers then built a new society, a Republic, based on the principles of liberty and justice for all.

(1)

The Republic of the United States has a long and rich history. It has been a land of freedom and opportunity for many people. It has been a land where people have come from all over the world to seek a better life. The Republic has grown from a small colony to a great nation. It has been a land of progress and innovation. It has been a land where people have made great discoveries and inventions. The Republic has been a land of peace and harmony. It has been a land where people have lived together in peace and harmony for many years. The Republic has been a land of hope and dreams. It has been a land where people have dreamed of a better future. The Republic has been a land of love and compassion. It has been a land where people have loved and compassionated each other. The Republic has been a land of courage and bravery. It has been a land where people have shown courage and bravery in the face of adversity. The Republic has been a land of wisdom and knowledge. It has been a land where people have gained wisdom and knowledge from their experiences. The Republic has been a land of faith and belief. It has been a land where people have had faith and belief in their future. The Republic has been a land of hope and dreams. It has been a land where people have dreamed of a better future. The Republic has been a land of love and compassion. It has been a land where people have loved and compassionated each other. The Republic has been a land of courage and bravery. It has been a land where people have shown courage and bravery in the face of adversity. The Republic has been a land of wisdom and knowledge. It has been a land where people have gained wisdom and knowledge from their experiences. The Republic has been a land of faith and belief. It has been a land where people have had faith and belief in their future.

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in the case of L-Mengo (see Figure 2.2).

Crude pools of S- and M-Mengo, prepared from L cell lysates as described in Routine Materials and Methods, had sufficiently high titers (10^8 PFU/ml or more) to be used without further concentration, but the available L-Mengo pools were not concentrated enough (6×10^6 - 4×10^7 PFU/ml) and a partially purified preparation was substituted. This more concentrated material had been subjected to a process involving precipitation of the virus with methanol at 4° , and a brief incubation of the resuspended pellet with trypsin and ribonuclease at 37° , followed by differential centrifugation. It contained about 10^9 PFU/ml, and was diluted tenfold in growth medium minus calcium before use.

Experimental design

Aliquots of L cells, containing 10^7 cells, were incubated in suspension at 37° with 10 ml of growth medium minus calcium (spinner medium) containing 10^8 PFU/ml of S-, M-, or L-Mengo. After 30 minutes, the cells were spun down and washed twice in warm medium to remove unattached virus. The cell pellets were then resuspended in 20 ml of spinner medium, and were transferred to small spinner flasks fitted with side arms and a magnetic stirring device, and containing 80 ml of spinner medium at 37° . One ml samples were immediately taken for estimation of infectious centers, after dilution, by the method of Ellem and Colter (1960), in order to ascertain what percentage of cells had been infected. At intervals thereafter, 2.0 ml aliquots of the cell suspensions were withdrawn. Exactly one ml of each aliquot was added to 4.0 ml of cold

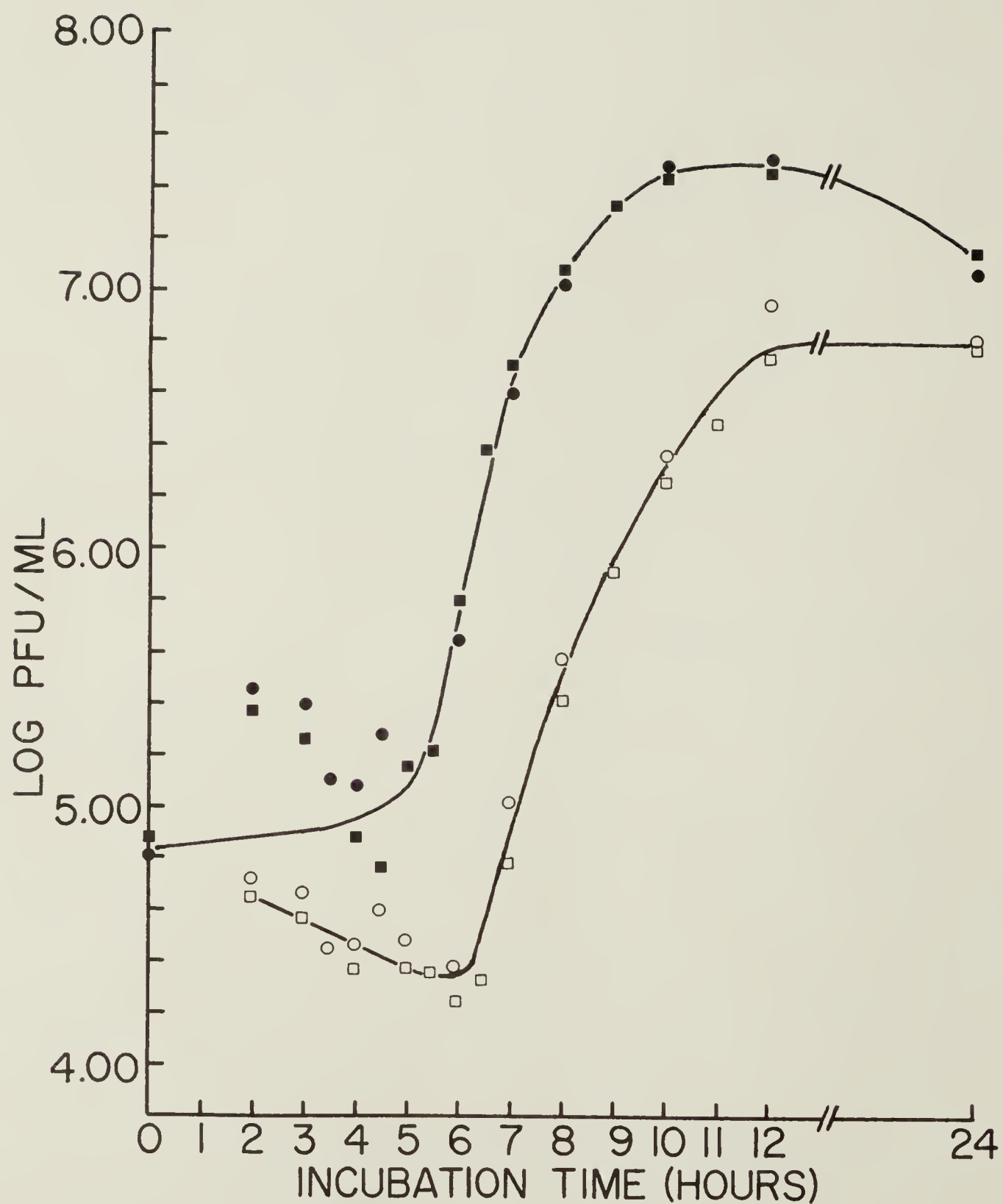


Figure 1.1. Single cycle growth curves of S-Mengo in L cells in suspended culture. Temperature of incubation = 37° ; original cell concentration = 100,000 cells/ml in spinner medium; input virus multiplicity = 100. Upper curve: total virus (zero time points: number of infected cells). Lower curve: extracellular virus.

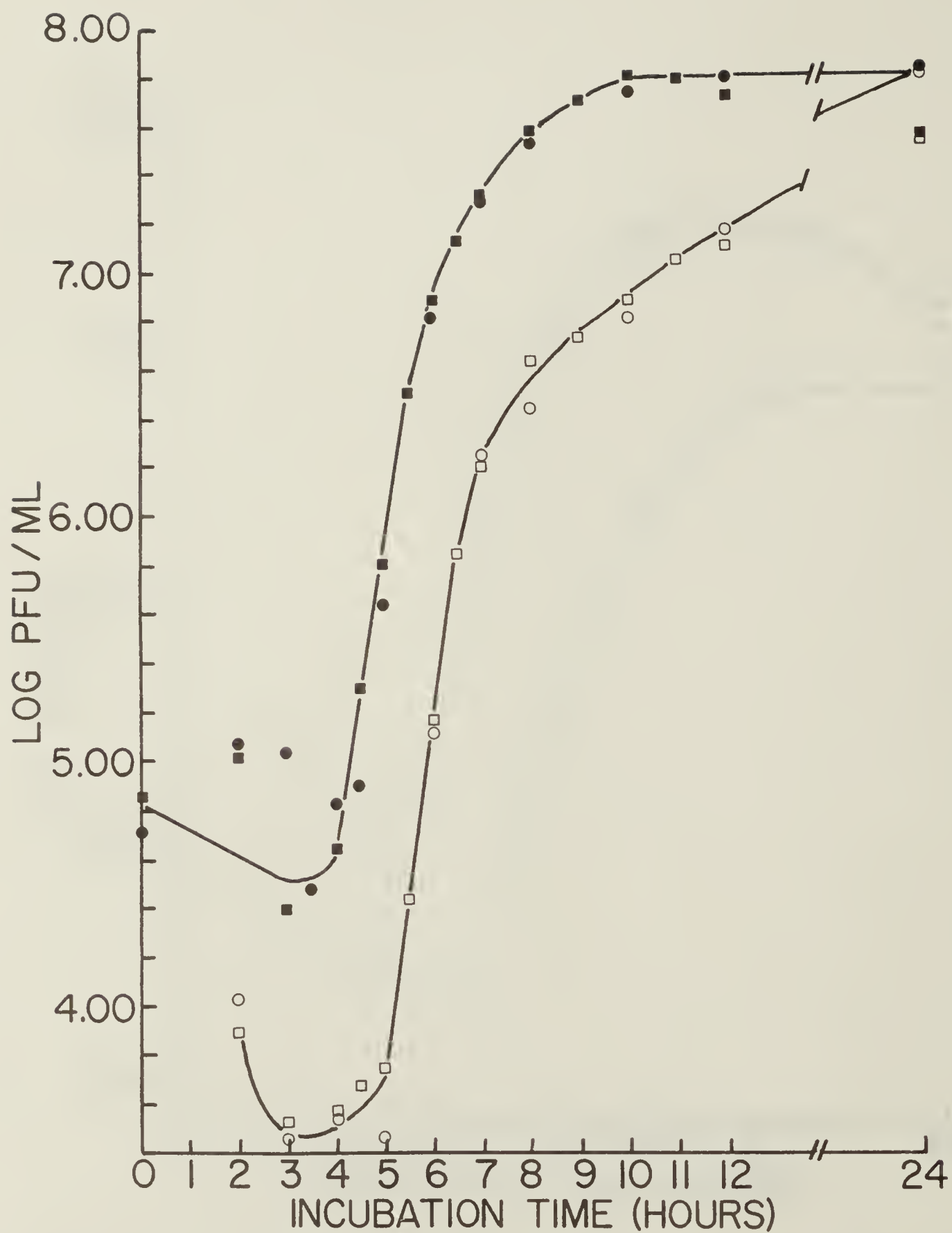


Figure 1.2. Single cycle growth curves of M-Mengo in L cells in suspended culture. Temperature of incubation = 37° ; original cell concentration = 100,000 cells/ml in spinner medium; input virus multiplicity = 100. Upper curve: total virus (zero time points: number of infected cells). Lower curve: extracellular virus.

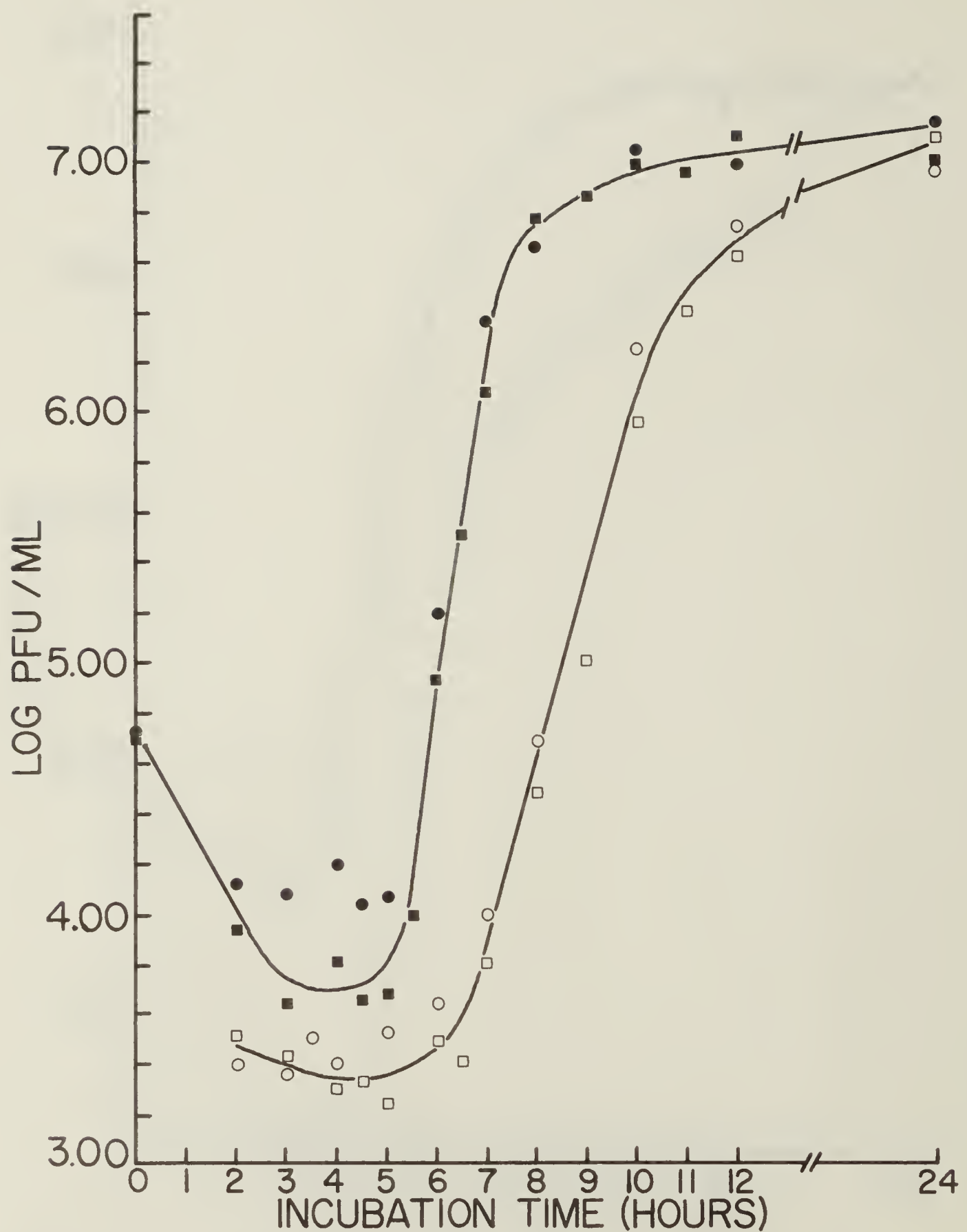


Figure 1.3. Single cycle growth curves of L-Mengo in L cells in suspended culture. Temperature of incubation = 37°; original cell concentration = 100,000 cells/ml in spinner medium; input virus multiplicity = 100. Upper curve: total virus (zero time points: number of infected cells). Lower curve: extracellular virus.

virus diluent, pH 7.6, subjected to a cycle of freezing and thawing, and was then stored at -20° . The remaining 1.0 ml was added to another 4.0 ml of cold virus diluent, which was then centrifuged to remove particulate material, and the supernatant was stored at -20° . This procedure allowed an estimate to be made, when the samples were later titrated, of the total virus and the extracellular virus present at each time interval. Since the 2.0 ml aliquots were removed from the spinner flasks through one of the side arms, there was very little loss of CO_2 from the internal atmosphere, and the pH did not alter significantly over the 24 hour period during which samples were taken.

Results and Discussion

The results of duplicate determinations of the growth curves of S-, M-, and L-Mengo in L cells are summarized in Figures 1.1, 1.2, and 1.3 respectively. There are several features of interest. Both in the time of maturation of virus and in the time of release of virus from the cells, the curves of L- and S-Mengo are similar. The eclipse phase is about 1.5 hours shorter with M-Mengo however, - 4-4.5 hours, as opposed to 5.5-6 hours with the other two variants, - and extracellular virus also appears earlier with this variant. It is evident that the large size of plaques produced by L-Mengo cannot be explained on the basis of a faster release of intracellular virus.

The scattering of points, and the apparent production of virus during the eclipse phases of the growth curves of S- and M-Mengo is puzzling, but may be due to elution of virus

from the cells. With an input virus multiplicity of 100, it is very likely that considerably more than one virus particle is adsorbed to each cell. With poliovirus, it has been shown that a considerable proportion of virus adsorbed to cells does not penetrate, but rather elutes (Joklik and Darnell, 1961; Fenwick and Cooper, 1962). Data presented in Chapter 2 indicates that this phenomenon occurs with the S and M variants, but not with L-Mengo. This may therefore explain why the eclipse phase of L-Mengo only shows a drop in the amount of detectable virus (Figure 1.3).

If then, elution of infectious virus is the cause of the apparent increase in virus titer noted during the first 3-4 hours of S- and M-Mengo virus growth, this may affect the accuracy of determinations of the burst sizes of these variants. Since the 'eluted' virus appears to be either inactivated or reabsorbed, however, it is unlikely that the degree of inaccuracy imposed by this factor is at all marked. Table 1.1 lists the calculated burst sizes of the three variants,

Table 1.1

Burst Sizes of the Mengo Variants in L Cells

Variant	PFU/cell	
	Expt. 1	Expt. 2
S	480	360
M	1200	890
L	200	240

The first part of the paper discusses the importance of the study and the objectives of the research. It also mentions the scope of the study and the limitations. The second part of the paper discusses the methodology used in the study. It mentions the data sources and the statistical methods used. The third part of the paper discusses the results of the study. It mentions the findings and the conclusions. The fourth part of the paper discusses the implications of the study. It mentions the policy implications and the future research.

TABLE 1

Summary of the results of the study

Variable	Mean	Standard Deviation	Minimum	Maximum
Age	35.2	12.5	20	50
Gender	Male	Female	Male	Female
Education	High School	College	University	Postgraduate
Income	Low	Medium	High	Very High

calculated from the data in Figures 1.1 - 1.3, on the basis of the amount of virus produced by 12 hours after infection. As implied by the high titers of M-Mengo pools and the relatively low ones of L-Mengo pools, M-Mengo produces considerably more infectious virus per cell than S-Mengo, and L-Mengo produces least of all.

Franklin (1962) described the growth characteristics of a small plaque variant of Mengo virus which mutated with a rather high frequency to a large plaque variant. When this small plaque variant was grown in L cell monolayers, new infectious virus appeared between 2.5 and 3.5 hours after initial infection, and increased in amount up to 6 hours. Extracellular virus appeared after 3.5 hours, but even at 12 hours after infection approximately 70% of the infectious virus was still intracellular. The average burst size was found to be 900 PFU/cell, although in individual experiments, yields of up to 12,000 PFU/cell were obtained. Dales and Franklin (1962) published growth curves of Mengo and EMC virus which were similar to those presented by Franklin (1962). Brownstein (1961) and Brownstein and Graham (1961) described a heat-resistant variant of Mengo virus (designated strain 37A) which produced small, ragged plaques of 1-2 mm in diameter after 2 or 3 days incubation in L cell monolayers under Noble agar overlay. This variant had an eclipse period of about 4 hours, and release of virus from the cells was complete by 7 hours after infection. The burst size was found to be about 500 PFU/cell. In later studies of the same system, the eclipse period was found to be 6.5 hours, and virus formation

was not complete until 9 hours post-infection (Homma and Graham, 1963a). In view of the fact that the eclipse periods of S-, M-, and L-Mengo differ quite markedly, the change in the system studied by Graham and coworkers (Brownstein and Graham, 1961; Homma and Graham, 1963a) may be due to mutation of the original 37A strain to another variant which does not differ too markedly from the original with respect to the sizes of plaques it produces in L cells under agar overlay.

The Mengo variants of both Franklin (1962) and Brownstein and Graham (1961) appear to have similarities to M-Mengo, but do not appear to be identical to it. It is difficult to draw exact comparisons, however, since differences in the composition of the growth and overlay media used by these workers may have a considerable influence on the growth characteristics of their variants in L cells. This difficulty does not arise in comparisons of the growth curves of the S-, M-, and L-Mengo variants, since these were determined at the same time and under exactly the same conditions. It is probable, therefore, that the differences observed between these three variants do exist. The main problem is to explain why they occur.

The reason for the low burst size of L-Mengo, as compared with M-Mengo, can at present only be a matter for speculation, although some explanations seem more likely than others. The possibility that the L variant multiplies to produce infective progeny in only a small proportion of the L cell population seems unlikely in view of the fact that the viable cell count of an L cell suspension is usually reduced to less than 10% of

its original value after incubation with L-Mengo for 24 hours. Preliminary heat-inactivation studies have indicated that all three variants have a half-life of the order of 8 hours at 37⁰, and there is no indication from the growth curves presented here, over the period of 12-24 hours post-infection, that L-Mengo is more rapidly inactivated than the other two variants. This possibility also, then, appears to be eliminated.

The number of infectious virus units produced per cell may have no bearing on the total number of virus particles formed. For example, the 37A strain of Mengo has been estimated to have a total:infectious particle ratio of 5 (Homma and Graham, 1963b) and ratios for the closely-related EMC and Maus-Elberfeld viruses have been given as 10 and 4000 respectively (Faulkner et al., 1961; Rueckert and Schäfer, 1965). Indirect evidence has suggested that the ratio of total particles:infectious particles produced per cell may be considerably higher with L-Mengo than with the S or M variants. In particular, the amount of infectious viral RNA which can be extracted per PFU of L-Mengo is considerably higher than that which can be extracted per PFU of the other two variants (Colter et al., 1962, and unpublished observations), which implies that L-Mengo pools contain a higher proportion of phenotypically incompetent, but genotypically competent, virus particles than those of S- or M-Mengo. Verification of this point, however, will require accurate estimations of the total:infectious particle ratio of each variant.

CHAPTER 2

Rate of Attachment to L Cells, and Effect of pH on Infectivity

Introduction

Clearly, the data presented in Chapter 1 to show that L-Mengo has the lowest burst size of the three variants do nothing to explain why this variant produces the largest plaques in L cell monolayers under agar overlay. Among the first of the studies carried out in an effort to uncover the reason for this readily demonstrable difference in the three variants were experiments designed to determine their rates of attachment to L cells. It was reasoned that if, of the three variants, L-Mengo had the highest affinity for L cells, this might account for its faster spread in the L cell monolayer with the resulting production of larger plaques. Even preliminary results, however, were sufficient to show that this was not the case. Arising from the rate of attachment study was an investigation of the effect of pH on the infectivities of the variants. Although neither study provided an explanation for the observation that the variants produce plaques of widely differing sizes, they did yield some very intriguing data, summaries of which are presented here.

Materials and Methods

Poliovirus strains. The six strains of poliovirus used--W.CHAT 26, Mahoney, Wistar II, MEF 1, WM-III, and Habel 24--were kindly supplied by Dr. Hilary Koprowski, The Wistar Institute, Philadelphia, Pennsylvania. Pools were

prepared in HeLa cells grown in suspended culture.

Buffers and virus diluents. The basic buffered balanced salt solution used throughout these investigations was that described by Dulbecco and Vogt (1954). In those studies in which the effect of pH on the infectivity of the variants was under examination, phosphate (0.01 M) was used as the buffer for the pH range 6.0 to 7.4, while for the pH range 7.2 to 8.6, TRIS buffer (0.01M) was substituted for phosphate. These solutions are referred to, in what follows, as PBS and TBS. In the case of the TBS solutions, the concentration of the sodium and potassium chlorides were increased slightly so that the ionic strength of all the buffered salt solutions was identical. Virus diluents were PBS or TBS to which bovine plasma albumin was added to a final concentration of 0.2 per cent.

Results

Rate of attachment of the Mengo variants to L cells

Monolayer system. Aliquots of suspensions of the three variants (containing known numbers of PFU in virus diluent of pH 7.4) were pipetted onto replicate cultures of L cells previously washed with PBS, pH 7.4. After incubation for periods of time ranging from 5 to 90 minutes, unattached virus was removed by washing the monolayers twice with 5 ml aliquots of PBS, and regular agar overlay was applied. Plaques were visualized and counted three days later.

The results of a large number of experiments are summarized in Figure 2.1. In order to pool the data from many experiments, the number of plaques produced at each time

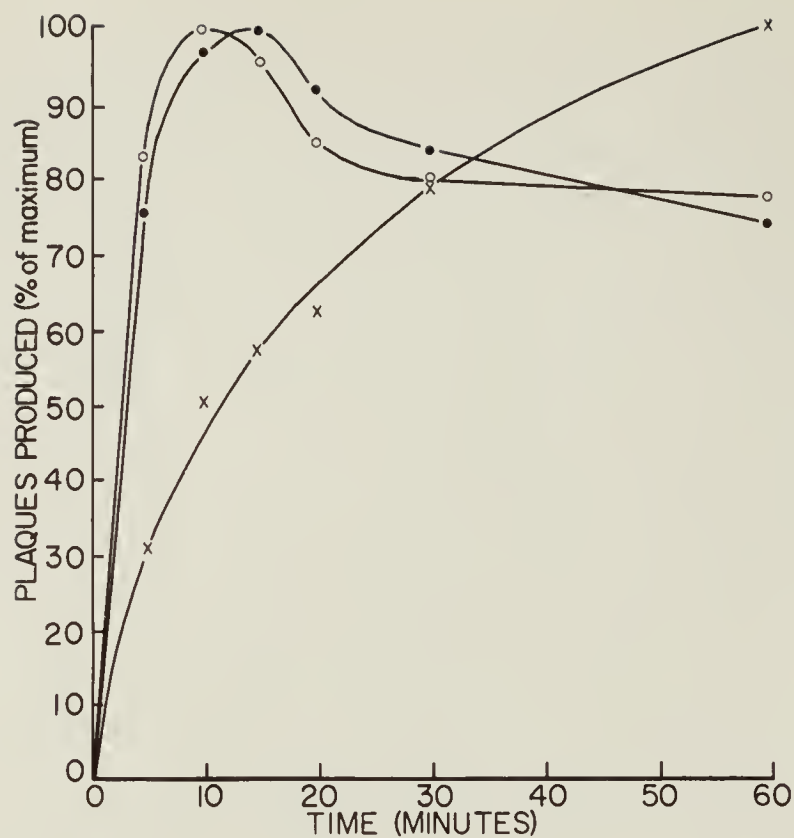


Figure 2.1. Rate of infection of L cell monolayers by the Mengo variants. ● = S-Mengo; o = M-Mengo; x = L-Mengo.

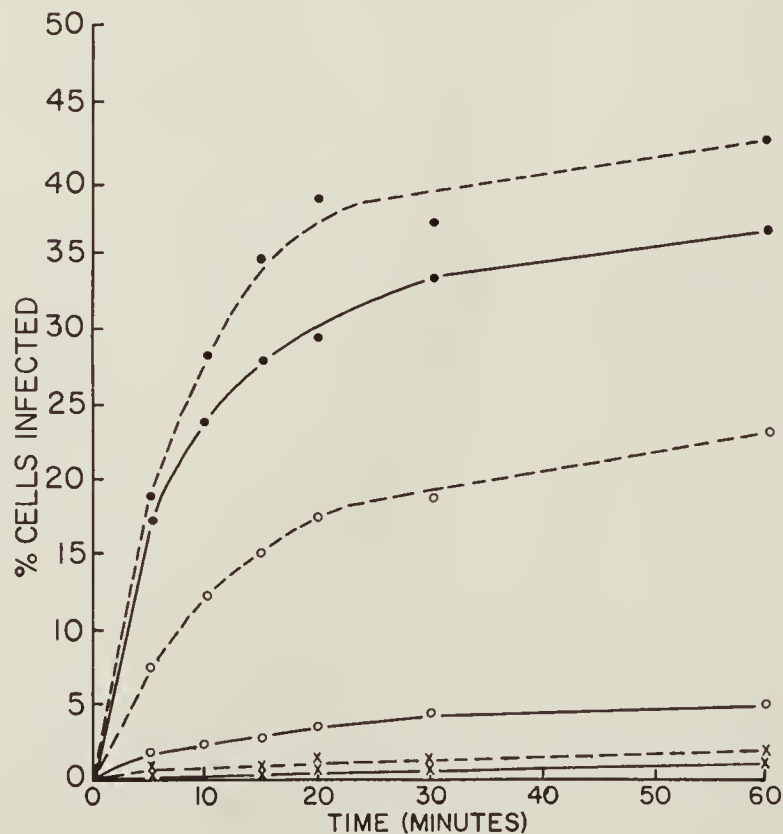


Figure 2.2. Rate of infection of suspended L cells by the Mengo variants. Solid lines: 100,000 cells/ml; input virus multiplicity = 5. Broken lines: 200,000 cells/ml; input virus multiplicity = 5. ● = S-Mengo; o = M-Mengo; x = L-Mengo.

interval in each experiment was expressed as a percentage of the maximum number formed in that experiment. Averages were then taken of the values obtained for each time interval and were used to construct the curves shown here.

Two features of this figure are worth noting. First, it is clear that the L variant attaches to L cells at a slower rate than do the M and S variants. Secondly, whereas the number of plaques produced by a suspension of L-Mengo increases progressively from time zero to a near maximum at 60 minutes, the curves relating plaques produced to time of incubation for the M and S variants show maxima at 10 and 15 minutes respectively. This somewhat surprising result was quite reproducible, and is reminiscent of the "sloughing" of poliovirus particles from HeLa and ERK cells described by Joklik and Darnell (1961) and Fenwick and Cooper (1962), respectively.

Suspended cell system. Aliquots of L cells, at a concentration of either 100,000/ml or 200,000/ml, were incubated with the variants in growth medium containing an input virus multiplicity of 5. At intervals, aliquots were removed and diluted tenfold with growth medium. The cells were sedimented, freed from unadsorbed virus by washing, and the number of infected cells per sample determined by titration on indicator monolayers of L cells (Ellem and Colter, 1960).

Figure 2.2 illustrates the results of a number of these experiments, in which the rate of formation of infectious centers in L cell--Mengo virus suspensions was measured. It is clear that here also, sizeable differences exist in the rates at which the variants attach to cells, i.e. in the

Table 2.1

Rates of Attachment* of Mengo Variants to L Cells

Variant	System	Cell concentration (cells/ml)	kc	k (cm ³ /cell/min)
S	Suspended cell	1 x 10 ⁵	0.124	12.4 x 10 ⁻⁷
S	Suspended cell	2 x 10 ⁵	0.116	5.8 x 10 ⁻⁷
S	Monolayer	6 x 10 ⁷	0.277	4.6 x 10 ⁻⁹
M	Suspended cell	1 x 10 ⁵	0.075	7.5 x 10 ⁻⁷
M	Suspended cell	2 x 10 ⁵	0.071	3.6 x 10 ⁻⁷
M	Monolayer	6 x 10 ⁷	0.355	5.9 x 10 ⁻⁹
L	Suspended cell	1 x 10 ⁵	0.049	4.9 x 10 ⁻⁷
L	Suspended cell	2 x 10 ⁵	0.046	2.3 x 10 ⁻⁷
L	Monolayer	6 x 10 ⁷	0.074	1.2 x 10 ⁻⁹

*Calculated from the equation $v_5 = v_{\infty}(1 - e^{-5kc})$, in which v_5 = PFU adsorbed in 5 minutes, v_{∞} = maximum number of PFU adsorbed, and c = cell concentration/ml.

relative affinities of the variants for cells. In the suspended cell system, the S variant attaches more rapidly than the M, and the M variant more rapidly than the L.

Actual rates of attachment of the Mengo variants to L cells estimated from data obtained from the two (monolayer and suspended cell) systems are given in Table 2.1. Only initial rates have been calculated, since the adsorption processes, at least in the present systems, do not, in general, appear to follow simple kinetics. The values calculated from data obtained with the monolayer system are of the same order of magnitude as those reported by other workers for other virus-cell monolayer systems: mouse encephalomyelitis virus-L cells (Henry and Franklin, 1959); poliovirus-HeLa cells and Cocksackie B1-HeLa cells (Holland, 1962); NDV-chick embryo lung cells (Rubin et al., 1957). The values arising from studies of the suspended cell system, however, differ by a factor of roughly 10 from those reported by Brownstein and Graham (1961) for a heat-stable variant of Mengo encephalomyelitis virus. These workers also reported that neither cell concentration nor virus concentration had a marked effect on the rate of attachment of this variant to L cells. This observation is at variance with those described here, since, in the suspended cell system at least, the rate constant is significantly affected by alterations in cell and virus concentrations.

Effect of pH on the infectivity of the Mengo variants

Monolayer system. In order to construct precise curves relating the infectivities of the Mengo variants to the pH at which the cells and virus were allowed to interact, a series

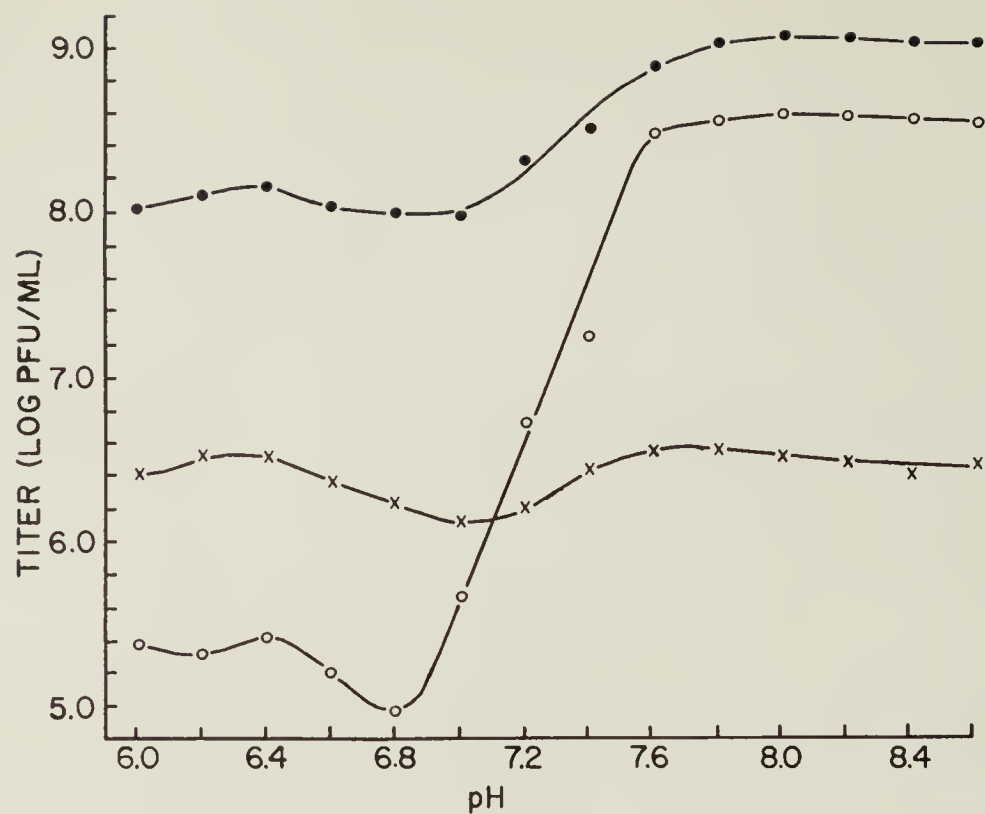


Figure 2.3. Effect of pH on the infectivity titers of the Mengo variants as measured by plaque assay. ● = S-Mengo; o = M-Mengo; x = L-Mengo.

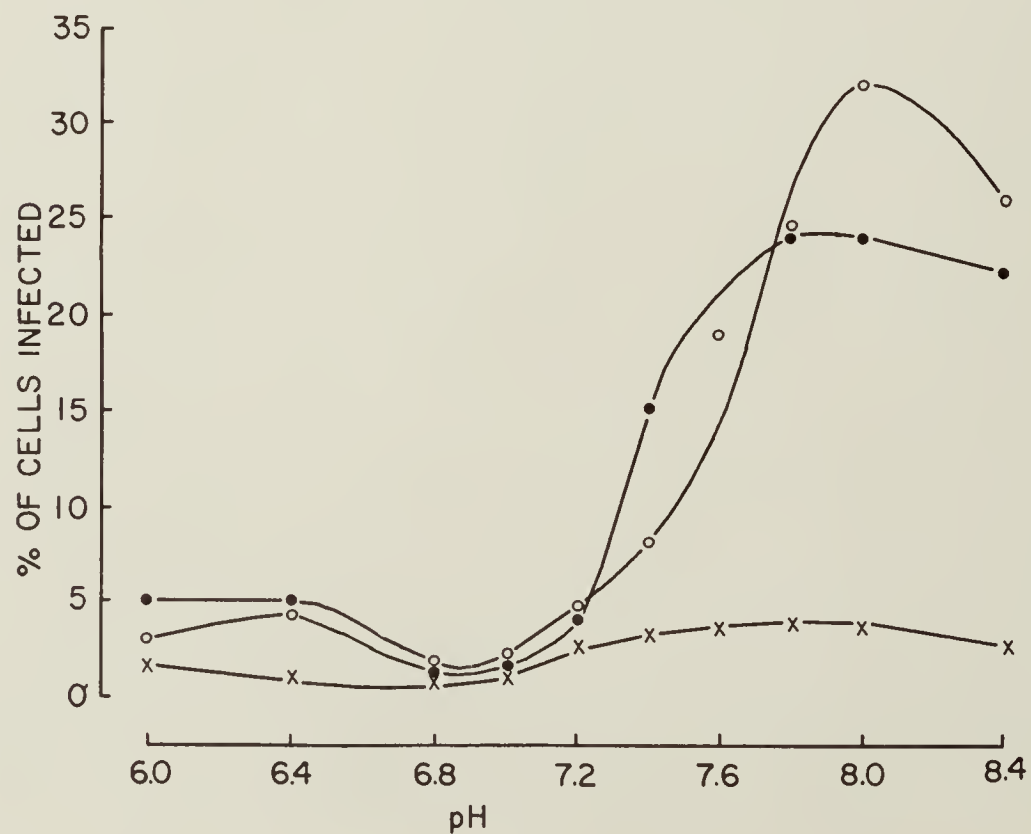


Figure 2.4. Effect of pH on the formation of infectious centers in L cell--Mengo variant suspensions. ● = S-Mengo; o = M-Mengo; x = L-Mengo.

of buffers and matching virus diluents were made to cover the pH range from 6.0 to 8.6 at intervals of 0.2 pH units, as described in the Materials and Methods section. Monolayers of L cells were carefully washed with PBS or TBS solutions covering the pH range, and after washing, virus was applied to the plates in virus diluent of the same pH as that of the salt solution used for washing. After incubation for one hour at room temperature (approximately 25⁰), the monolayers were again washed with balanced salt solution of the appropriate pH, regular agar overlay was applied, etc. It is perhaps worth noting that in each experiment, the PBS and the TBS series overlapped at pH's 7.2 and 7.4. No evidence of a "break" in the curve due to a change from phosphate to TRIS buffered solutions was ever noted. It should be emphasized too that the pH was manipulated during the period of virus attachment and penetration only. All plates were overlaid with the same agar overlay, and plaque development proceeded in all cases under the same conditions of pH.

The pooled results of a large number of experiments are illustrated graphically in Figure 2.3. The infectivity of the L variant is little affected by the pH at which cells and virus interact, that of the S variant is affected somewhat more, while that of the M variant is profoundly affected. Increasing the pH from 6.8 or 7.0 (the minimum point on all three pH-infectivity curves) to 8.0 increases the infectivity of the L, S and M variants by factors of 2, 10, and 3,000 respectively.

The data illustrated in Figure 2.3 were obtained from experiments utilizing single pools of each of the three variants. However, the possibility that the results reflected some peculiarity of these particular pools was ruled out. The same phenomenon was observed with a number of pools of each variant prepared over a period of many months.

Suspended cell system. This dependency on pH holds true in the suspended cell system as well, as may be seen from Figure 2.4. Here again, the results of a large number of experiments have been pooled. Aliquots of cells (10^6 cells) were washed in PBS or TBS, and were then resuspended in 5 ml of a virus suspension in virus diluent of the appropriate pH. The virus dilutions were chosen to give a multiplicity of infection of 5. After incubation for 30 minutes at room temperature, the suspensions were centrifuged, the cells washed twice with 5 ml aliquots of the corresponding PBS or TBS solution, and the number of infectious centers therein determined in the usual manner. In Figure 2.4, the results are expressed as the percentage of cells infected at each pH at which cell-virus mixtures were incubated.

Effect of pH on the infectivity of poliovirus

It seemed of interest to determine whether the dramatic effect of pH observed with two of the three Mengo variants would be seen in the interaction of other small neurotropic viruses and appropriate host cells. The effect of pH on the interaction of six strains of poliovirus (one virulent and one attenuated of each of the three polio types) and HeLa cells was examined, using the monolayer system. The data

shown in Table 2.2 make it clear that, over the range 6.6 to 8.0, the number of plaques produced by standard dilutions of these poliovirus strains is unaffected by the pH at which cells and virus interact.

Table 2.2

Effect of pH on Attachment of Poliovirus
Strains to HeLa Cell Monolayers

pH	Number of plaques/plate					
	Habel	MEF 1	WM-III	W.CHAT	Wistar	Mahoney
6.6	65	62	-	-	-	-
6.8	70	70	32	40	33	70
7.0	65	70	36	37	31	68
7.2	65	63	33	35	32	62
7.4	66	69	-	-	-	-
7.6	63	61	32	35	29	64
8.0	56	61	28	36	31	62

Mechanism of the pH effect

The most attractive hypothesis with which to explain the effect of pH on the infectivity of the S and M variants was that the pH-infectivity curves reflected what one might call the "titration" curve of the attachment sites on the virus particles. It seemed reasonable to assume that by changing the pH at which cells and virus interacted, the charged nature, and possibly the configuration, of the group or groups responsible for attachment were changed, and that the efficiency with which the virus attached to the host cell

Table 2.3

Effect of pH on the Interaction of L Cells
in Suspension with S- and M-Mengo:
Recovery of Added Virus

Sample	Incubation period (mins.)	I.C.*/ml incubation mixture	PFU/ml supernatant	I.C.* + PFU/ml
S-Mengo, pH 6.8	15	5.2×10^3	13.0×10^3	18.2×10^3
"	30	4.6×10^3	3.6×10^3	8.2×10^3
"	60	3.6×10^3	1.4×10^3	5.0×10^3
S-Mengo, pH 8.0	15	3.6×10^4	9.2×10^4	12.8×10^4
"	30	4.4×10^4	7.1×10^4	11.5×10^4
"	60	5.0×10^4	3.9×10^4	8.9×10^4
M-Mengo, pH 6.8	15	1.8×10^3	5.5×10^3	7.3×10^3
"	30	2.0×10^3	3.2×10^3	5.2×10^3
"	60	1.8×10^3	1.1×10^3	2.9×10^3
M-Mengo, pH 8.0	15	4.8×10^4	1.5×10^6	1.5×10^6
"	30	6.6×10^4	1.4×10^6	1.5×10^6
"	60	6.6×10^4	1.2×10^6	1.3×10^6

* Infectious centers.

All incubation mixtures contained 200,000 cells/ml. Input multiplicity: S-Mengo = 1; M-Mengo = 5. Incubation at 25°.

was thereby altered. It would be predicted on this basis that in a cell-virus mixture incubated at pH 6.8--in which very few of the cells became infected--the bulk of the virus would remain unattached and would be detectable after the cells were removed by centrifugation. This turned out not to be the case.

Aliquots of L cells were incubated with the M and S variants at pH's 6.8 and 8.0. At intervals, aliquots of the suspensions were removed, and the cells separated by centrifugation. The number of infectious centers in the cell samples and the number of PFU of virus remaining in the supernatants were determined.

It may be seen from Table 2.3 that in those mixtures incubated at pH 6.8, not only were very few cells infected, but very few infectious virus particles could be detected in the supernatants. Most of the input virus particles could not be detected, either as cell-associated or unattached virus.

This prompted an examination of the effect of pH alone--that is, in the absence of cells--on the infectivity of the M and S variants. The two variants were suspended as 1:10 dilutions of the respective pools in balanced salt solutions at pH's 6.8, 7.4 and 8.0. After incubation at room temperature for one hour, additional dilutions of each sample were carried out in virus diluent of pH 7.6, and the number of PFU of virus remaining in each was determined. It was found that the M variant had been extensively inactivated at pH 6.8, and that the S variant had undergone a lesser, but still significant inactivation at that pH. More recent studies have shown that both variants are stable at pH's of 7.2 - 8.0, and that

both are inactivated at an increasingly rapid rate as the pH is lowered from 7.2 to 6.0.

Effect of tonicity on the infectivity of Mengo virus and poliovirus

In studies of the uptake of Mengo RNA by L cells using a suspended cell system, Ellem and Colter (1960) found that any increase in NaCl concentration above physiological virtually abolished the interaction of intact virus and cells. The possibility was examined, then, that incubation of virus with cell monolayers in hypotonic media might lead to an increased uptake of virus.

A simplified form of virus diluent, consisting of 0.02M potassium phosphate buffer, pH 7.0, containing 0.2% BPA and concentrations of NaCl varying from 0.02 to 0.42M, was used as the suspending medium in these experiments. When cells were incubated in solutions of NaCl concentration less than 0.02M, they usually died or became detached from their plastic substrate.

A stock virus dilution was prepared in normal virus diluent, and the two final tenfold dilutions were made from this in the test media. Duplicate monolayers were washed with appropriate media before the corresponding virus dilution was applied. After incubation for one hour at 25°, the small amount of fluid on the monolayers was removed by suction, the monolayers were washed once with growth medium, and overlaid with regular agar overlay and incubated as usual.

Data obtained from these experiments are illustrated in Figure 2.5. Decreasing the tonicity of the media resulted in

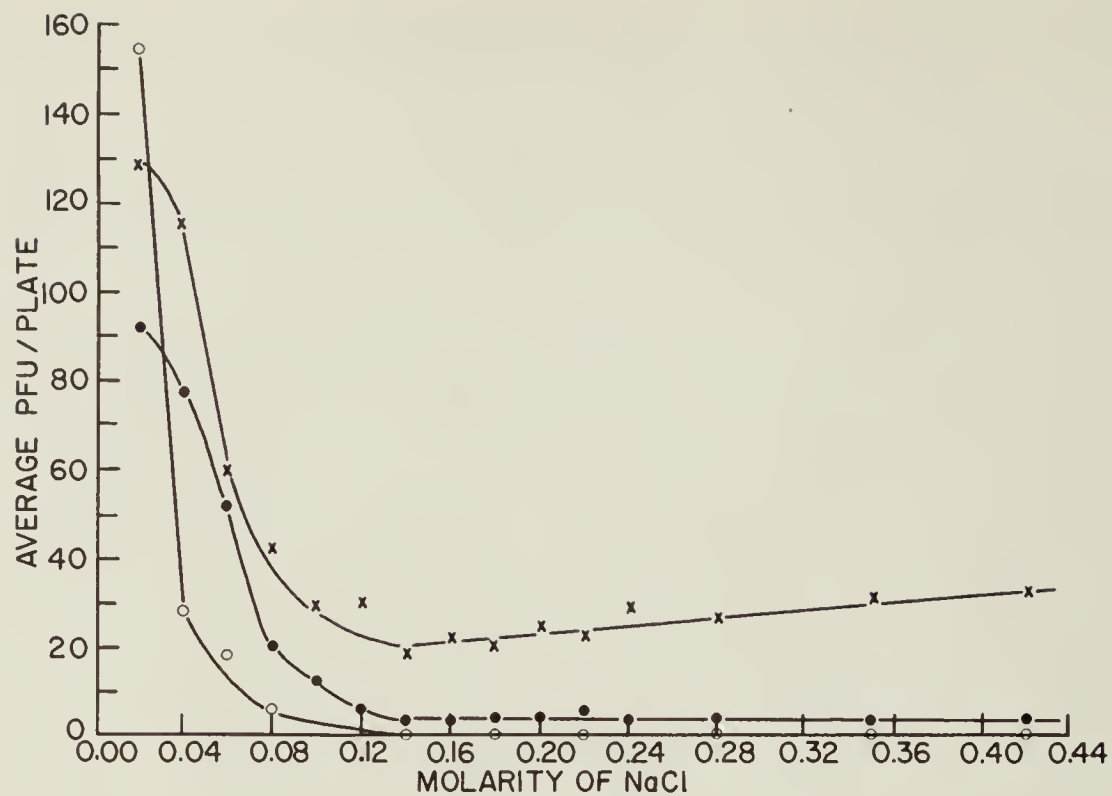


Figure 2.5. The effect of tonicity on the infectivity of the Mengo variants, as measured by plaque assay. ● = S-Mengo; ○ = M-Mengo; x = L-Mengo.

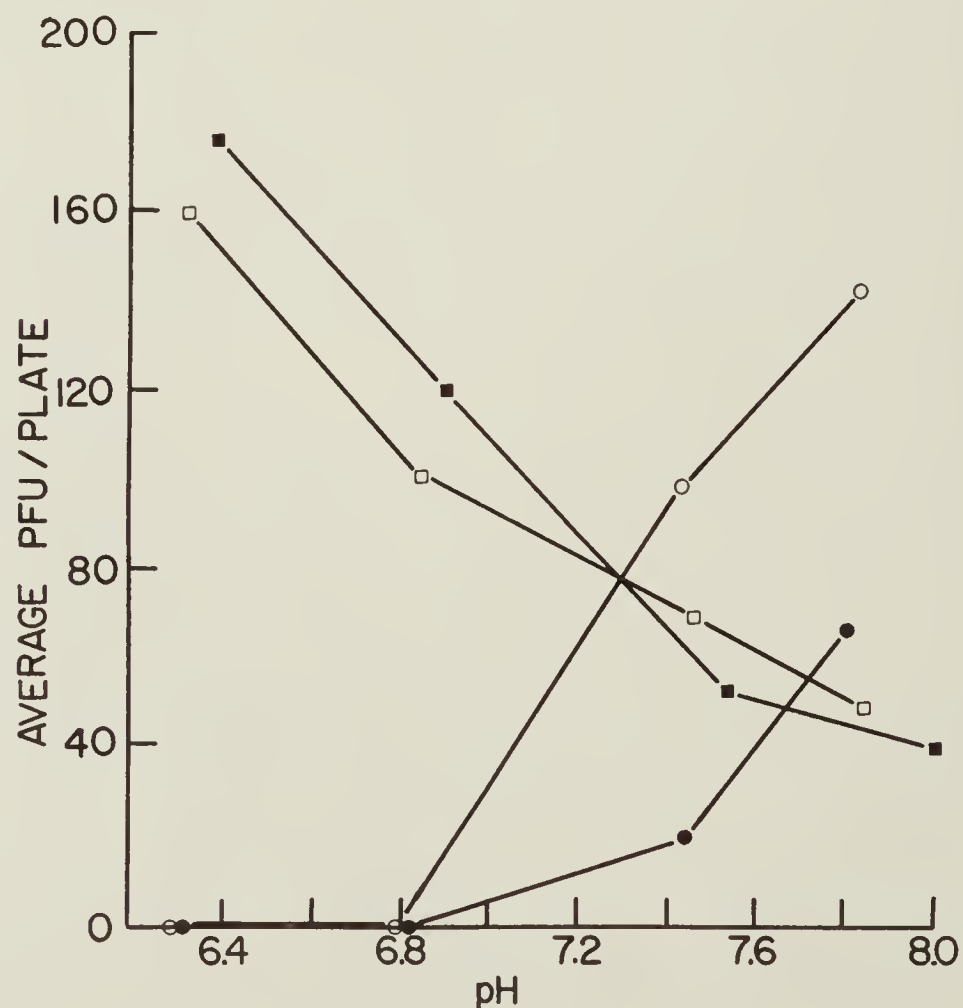


Figure 2.6. The effect of halides on the pH sensitivity of M-Mengo. The suspending media consisted of 0.02M phosphate buffer containing 0.2% BPA and 0.14M NaCl (○), NaBr (●), NaI (□), or NaNO₃ (■).

a considerable increase in the infectivity of all three Mengo variants. It is interesting to note that the magnitude of the relative increases is reminiscent of the extent to which each variant responds to pH changes (see Figures 2.3 and 2.4); i.e. M-Mengo is the most, and L-Mengo the least sensitive to changes in tonicity.

Similar experiments were carried out with the six polio-virus strains. Over the range of NaCl concentration tested, five of the strains showed little or no change in infectivity: the sixth (Mahoney) showed a 50% decrease of infectivity over the range 0.14M to 0.02M NaCl. These results are again reminiscent of the data arising from the pH studies (see Table 2.2).

Effect of halides on the pH sensitivity of Mengo virus

Boesche and Drees (1957) observed a marked instability of EMC and Columbia-SK viruses in media containing NaCl. This observation was extended by Speir et al. (1962) who found that Mengo virus and the MM strain of Columbia-SK virus lost infectivity at 37° in isotonic NaCl, but not in hypertonic (1M) saline or in distilled water. The presence of NaCl appeared also to contribute to the loss of infectivity of Mengo virus between pH 5 and 7 (Speir, 1962). It was therefore considered worthwhile to reexamine the effect of pH on the infectivities of the three variants, using media containing ions other than chloride.

The procedure utilized was the same as that used in the original pH studies (monolayer system). Solutions consisting of 0.14M NaCl, NaBr, NaI, or NaNO₃ in 0.02M potassium phosphate buffer containing 0.2% BPA were made up at 4 pH's: 6.3-6.4,

6.8-6.9, 7.4, and 7.8-8.0. These solutions were used to wash the monolayers of L cells and as diluents for the infecting virus. After incubation for one hour at 25°, the monolayers were washed, overlaid, and incubated as usual. The results from one such experiment with M-Mengo are presented in Figure 2.6. The enormous increase in infectivity of M-Mengo with increasing pH was found to be reduced when chloride was replaced by bromide, and in media containing NaI or NaNO₃ in place of NaCl, M-Mengo was shown to be considerably more infectious at pH 6.4 than at pH 7.8. Comparable experiments have not been carried out with the S and L variants, although a single one with L-Mengo, in which nitrate was substituted for chloride, gave a twofold decrease in infectivity over the pH range 6.5 to 7.9.

A preliminary study of the infectivities of the three variants in media containing a variety of electrolytes and non-electrolytes was also revealing. Identical virus dilutions were made up in the solutions listed in Table 2.4, and their infectivities were determined in the usual way on L cell monolayers prewashed with the appropriate media. The inhibitory effect of chloride on the infectivity of the three variants is evident from this table. It is also clear that the ionic composition of the suspending medium can affect considerably the infectivities of the Mengo variants.

Table 2.4

Effect of Solute Composition on Mengo Infectivity

Solute*	Average PFU/plate		
	S-Mengo	M-Mengo	L-Mengo
0.14M NaCl	37	2	37
0.14M NaI	146	124	43
0.07M Na glutamate	419	131	157
0.05M Na citrate	422	103	89
0.14M sucrose	211	138	148
0.14M glucose	214	156	163

* In 0.02M potassium phosphate buffer, pH 7.0, containing 0.2% BPA.

Discussion

An examination of the biological properties of the three variants considered here has not provided an explanation for the difference in size and morphology of the plaques that the agents produce in L cell monolayers. The observed differences in the rates at which the three variants attach to L cells do suggest, however, that they differ from one another with respect to the specific grouping (probably polypeptide in nature) responsible for their attachment to L cells.

This tentative conclusion is supported also by the observation that pH has a profound effect on the infectivity of the M and S variants, although the mechanism of the pH effect is

not a simple one. It is clear that much of the effect of pH on the infectivity of these two variants is due to their rapid inactivation at the lower pH's. It is not, however, the whole story, since there is no inactivation at pH 7.2, although this pH is significantly lower than that at which the agents are maximally infectious. Moreover, both variants are inactivated (in the absence of cells) more extensively at pH 6.0-6.4 than at pH 6.8, yet the latter is the minimum point on the pH-infectivity curves. The most likely explanation for the observations described here is that two phenomena are at work: inactivation of the M and, to a lesser extent, the S variant at pH's between 6.0 and 7.0, and an effect of pH on the charged nature and/or configuration of the attachment groups on the surface of the virus particles, which in turn affects the efficiency with which the agents attach to the host cell.

Although work to uncover the mechanism of inactivation of the variants at pH's less than 7.2 has not yet reached a definitive stage, certain observations are worth noting here. Firstly, it has been possible to extract infectious RNA by the method of Colter et al. (1957) from M- or S-Mengo incubated for one hour in buffered saline, pH 8.0, but not from virus incubated for the same period of time at pH 6.8. Secondly, highly purified virus, submitted to the same treatment, was inactivated to a lesser degree at the lower pH than were unpurified preparations.

On the basis of these results, the following working hypothesis has been enunciated to explain the inactivation of

M- and S-Mengo at low pH's. The proposition is that the protein coats of the pH-sensitive variants are altered in such a way that the viral genome becomes susceptible to degradation by cellular nucleases present in the crude virus pools. The different degrees of sensitivity of the three variants to inactivation at pH's less than 7 may be yet another manifestation of differences in the charge distributions of their protein coats. L-Mengo particles may have a lower net charge at low pH's than do the other two variants, and the subunits of its protein coat may thereby have little tendency to dissociate by mutual repulsion. Cellular nucleases would therefore have less ready access to L-Mengo RNA than to the genomes of the other variants, and this variant would be the most stable of the three at low pH's, which is what has been found.

The process of viral degradation at mildly acid pH has been observed with at least one other member of the Columbia-SK group--the Maus-Elberfeld (ME) virus. By incubating crystalline preparations of this virus with 0.7M NaCl, buffered with phosphate to pH 6.0, for two hours at 37⁰, Hausen et al. (1963) obtained protein-split products which had molecular weights of about 6×10^5 and 1×10^6 . No further details of this observation, however, were given.

The role of chloride in the inactivation process is obscure. Certainly it plays an important part, as seen from the data in Figure 2.6 and Table 2.4. It is also very probable that the increase in infectivity of the three variants in hypotonic solutions of NaCl (Figure 2.5) is due more to the reduction in chloride concentration than to the decrease in

tonicity. Experiments in which NaCl was replaced by sodium phosphate, pH 7.0, at concentrations ranging from 0.02 to 0.14M, gave rather erratic results, but they suggested that at low concentrations at least, chloride may have a stabilizing influence, and indicated that tonicity per se does not affect Mengo infectivity.

Speir (1961) and Speir et al. (1962) found that inactivation of Mengo virus in sodium halide salts occurred most extensively in chloride and bromide, less in iodide, and very little in fluoride, the pseudohalogen thiocyanate, or distilled water. They suggested that this might reflect a dependence of the inactivation on the size or degree of hydration of the anion. The data arising from studies of the effects of sodium chloride, bromide, and iodide on the pH sensitivity of M-Mengo (see Figure 2.6) are in accord with this suggestion. Nitrate and iodide, both large anions compared to chloride ions, gave similar curves. Fluoride could not be tested in the present system, since it rapidly killed the monolayer cells.

The reason for the "peaking" phenomenon observed in studies of the rate of attachment of S- and M-Mengo to L cell monolayers is not clear. Nor is it easy to understand why the effect should be absent in the suspended cell system. One might speculate that with these variants, the initial attachment is very rapid, and gives rise to a loose cell-virus complex from which the virus may either dissociate, or proceed to a more permanent association, in which case it would register as a PFU. In the monolayer method, the gentle

washing procedure may not dissociate the cell-virus complex, and the application of the agar overlay may "fix" virus particles that would otherwise have eluted from the cells. The more vigorous washing procedure used with the suspended cell system (centrifugations, resuspensions by means of pipette) on the other hand, may efficiently break all loose cell-virus complexes, and thus permit the measurement, at any time interval, of only those virus particles that had proceeded to the second stage,--the permanent association with the cell. An extension of this reasoning could provide an explanation for the apparent anomaly that M-Mengo attaches more rapidly than S-Mengo to monolayers, but less rapidly in the suspended cell system. The earlier "peaking" seen in the rate of attachment curve with this variant suggests that it may form the easily dissociable cell-virus complex somewhat more rapidly than does the S variant, while its apparently slower rate of attachment in the suspended cell system may reflect either a more readily dissociable complex than is formed with the S variant, or a slower rate of conversion of the initial complex to an undissociable form.

CHAPTER 3

The Pathogenicity of the Three Variants to Mice

Introduction

Data presented in the previous two chapters have shown that of the three Mengo variants under investigation, L-Mengo has the lowest burst size and attaches least readily to mouse L cells. In view of these observations, it was rather surprising to find that L-Mengo was by far the most virulent of the three variants when injected intraperitoneally into mice. The present section summarizes the results of a study of the distribution of the variants in mice following intraperitoneal injection, and records attempts to find an explanation for the high virulence of L-Mengo and the relative avirulence of the S and M variants.

Materials and Methods

Mice. Random-bred Swiss albino mice were used throughout this study. With the exception of the experiments involving suckling mice, in which both males and females were used randomly, only male mice were used. The mice, housed in stainless steel cages with bedding of sawdust and wood shavings, were fed on Purina laboratory chow (Labena) and water ad libitum.

Injection procedures. After swabbing the abdomens with 70% ethanol, mice were injected intraperitoneally with 0.5 ml of virus in virus diluent, using 2.5 ml disposable syringes equipped with 25 gauge needles. For intracerebral injection,

the mice were first anesthetized with ether. In this case, 1 ml disposable syringes equipped with 27 gauge needles were used, and each mouse received an injection of 0.03 ml.

Interferon production and assay. Viruses. Chikungunya virus, African strain, was kindly supplied by Dr. H. B. Levy, Bethesda, Md. It was passed twice in suckling mouse brain before use in the present work. Vesicular stomatitis virus (VSV) was obtained from the Wistar Institute, Philadelphia, through the courtesy of Dr. S. A. Plotkin. A pool was prepared from infected L cells by the same procedure as that used to prepare pools of Mengo virus.

Preparation of mouse interferon. The method of Levy (1964) was used. Heads and viscera were removed aseptically from 12-15 day mouse embryos, and the remainder was trypsinized and used to prepare primary fibroblast cultures in Blake bottles. When growth of the fibroblasts had produced confluent monolayers (2-3 days), the cell sheets were heavily infected with Chikungunya virus. After allowing the virus to adsorb for 2 hours at 37°, the monolayers were washed and incubated for another 20 hours with 80 ml of Eagle's medium (no serum). The supernatant fluid was then decanted, centrifuged to remove particulate material, and dialysed against 0.1M KCl/HCl, pH 2 at 4° in order to inactivate virus. After 24 hours at pH 2, the pH of the interferon-containing medium was adjusted to pH 7.4 by dialysis against PBS. A mock interferon control was prepared by processing media from uninfected monolayers in exactly the same way. The interferon preparation used in the present work reduced the plaque count of VSV

in L cells by 50% at a dilution of 1:180 when titered by the method described in the following paragraph. In experiments involving interferon production by Mengo virus, virus was inactivated at pH 2 for 3-4 days instead of 24 hours.

Interferon assay. Interferon was estimated by the plaque inhibition method. L cell monolayers were incubated for 18 hours at 37° with 2 ml aliquots of the interferon preparations diluted in growth medium. The fluid medium was then removed, and the monolayers were challenged with 40-80 PFU of VSV before being overlaid with regular agar overlay 1 hour later. Virus plaques were counted 2 days later after being visualized by staining the monolayers with neutral red. In addition to reducing plaque numbers, preparations containing interferon also reduced plaque sizes. This, however, was not taken into consideration in estimating the potency of the preparations, and all visible plaques were counted, regardless of size. All assays were done in duplicate or triplicate.

Results

Clinical symptoms following viral infection

Symptoms of viral infection became evident from the second day onward after inoculation, the time at which they appeared depending on the variant and the dosage given. Mice which had developed no symptoms by the 7th or 8th day usually recovered without showing any external signs of viral infection.

The course of Mengo infection in mice followed the pattern noted by Pérol-Vauchez et al. (1961) with EMC virus-infected mice. The illness first showed itself as a monoplegia,

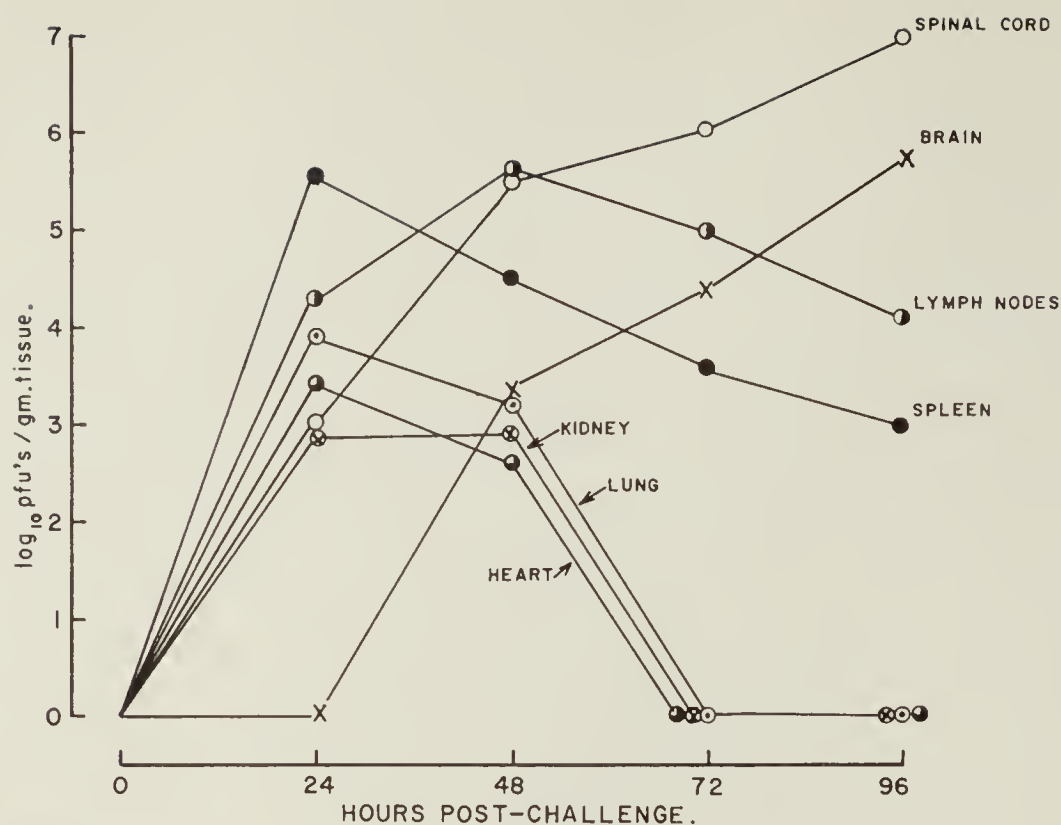


Figure 3.1. The distribution of infectious virus particles in the tissues of mice following the intraperitoneal injection of 50,000 PFU of L-Mengo.

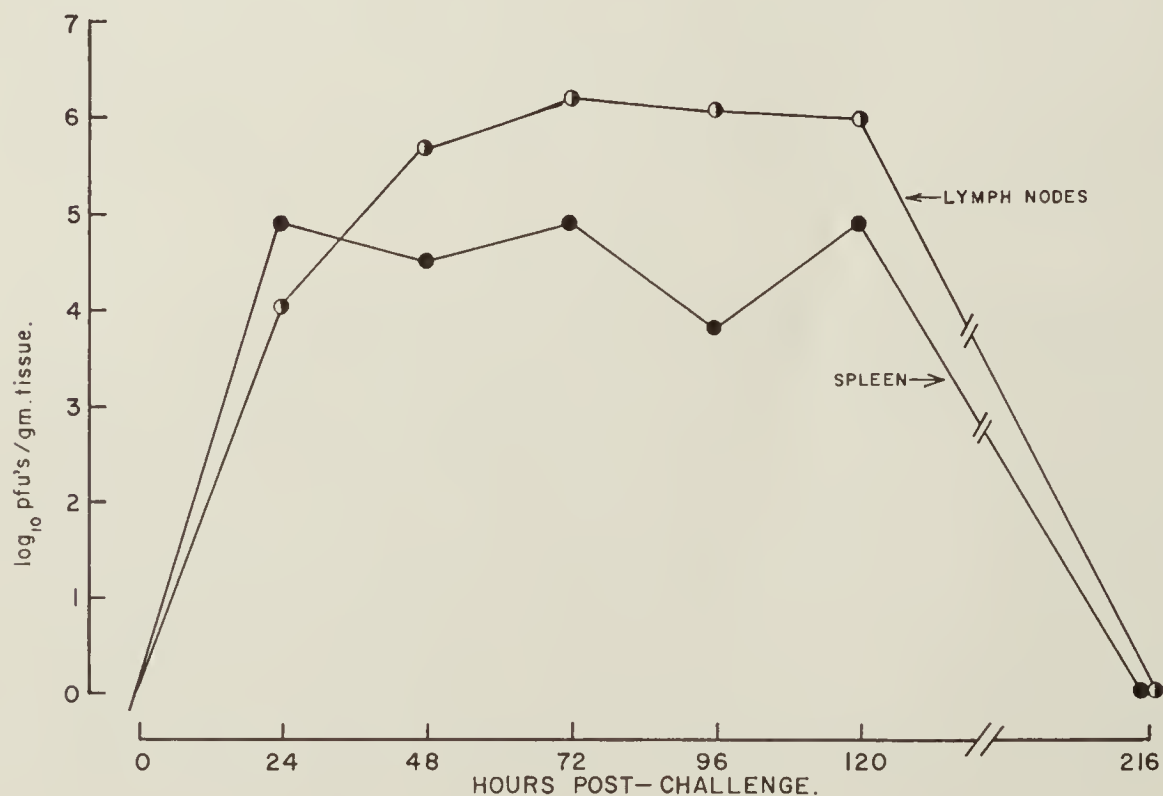


Figure 3.2. The distribution of infectious virus particles in the tissues of mice following the intraperitoneal injection of 50,000 PFU of M-Mengo. (An identical picture was obtained when 50,000 PFU of S-Mengo was injected).

most often in the hind limbs, or by a muscular atrophy marked by an arching of the back (paraplegia). Death, preceded often by an intense cyanosis, followed, - generally on the day after the appearance of the paralysis.

Fate of virus following intraperitoneal injection

In order to examine the behavior of the Mengo variants following intraperitoneal injection into mice, the following experimental design was chosen. A known number of PFU of virus was injected intraperitoneally into a group of mice. At intervals of 24 hours thereafter, two mice were killed, and their brains, spinal cords, hearts, lungs, livers, kidneys, spleens, inguinal lymph nodes, and blood removed. The tissues were homogenized separately as 10% suspensions in virus diluent, and the suspensions were clarified by a brief, low-speed centrifugation. The amount of virus in each supernatant was determined by titration in L cell monolayers. In these studies, young adult (24-26 g) male mice were used.

In the initial series of experiments concerning the fate of the Mengo variants following intraperitoneal injection into mice, a challenge dose of 50,000 PFU was arbitrarily chosen. The distribution of virus particles in the various mouse tissues following the administration of 50,000 PFU of L-Mengo is illustrated in Figure 3.1. At 24 hours post-challenge, spleen contained the highest level of virus. The level in this tissue decreased progressively thereafter. The amount of virus in the lymph nodes rose to a maximum at 48 hours and stayed at a fairly high level throughout the course of the experiment. In this particular experiment, virus was detectable

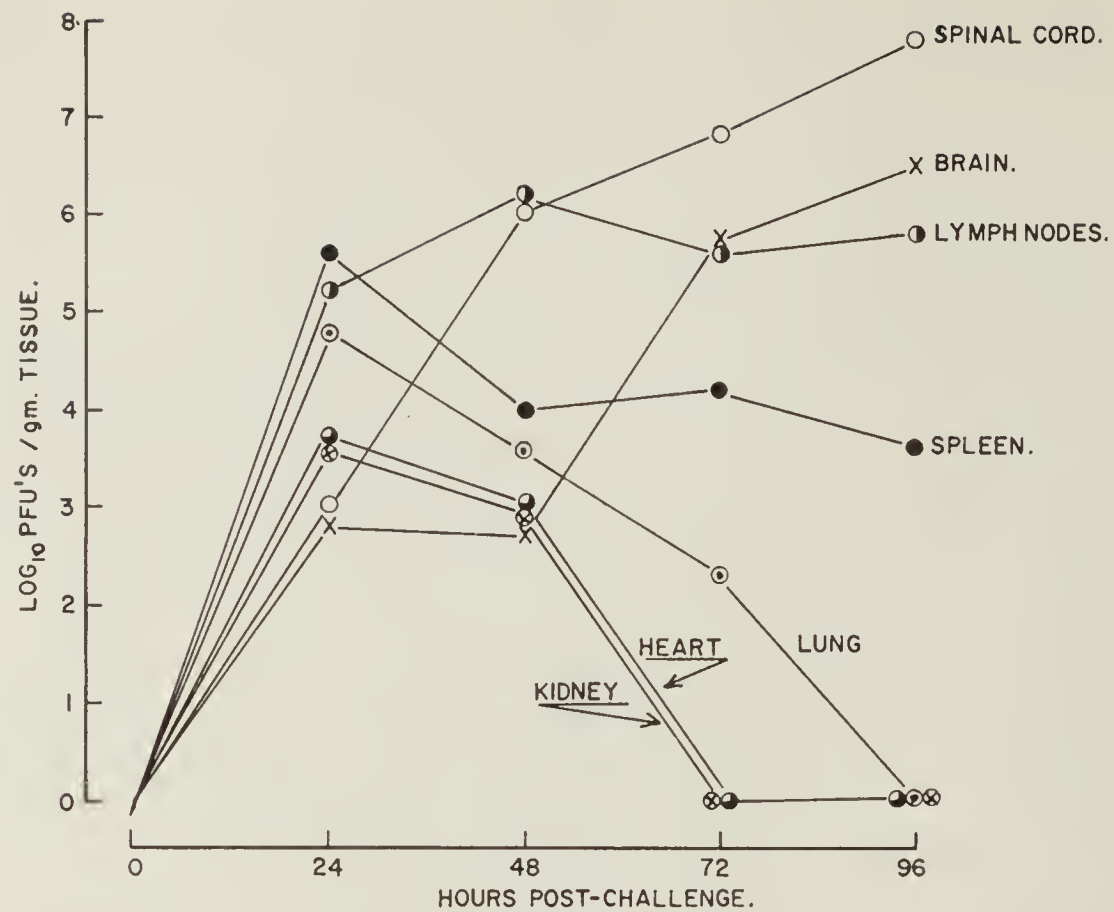


Figure 3.3. The distribution of infectious virus particles in the tissues of mice following the intraperitoneal injection of 5×10^7 PFU of M-Mengo.

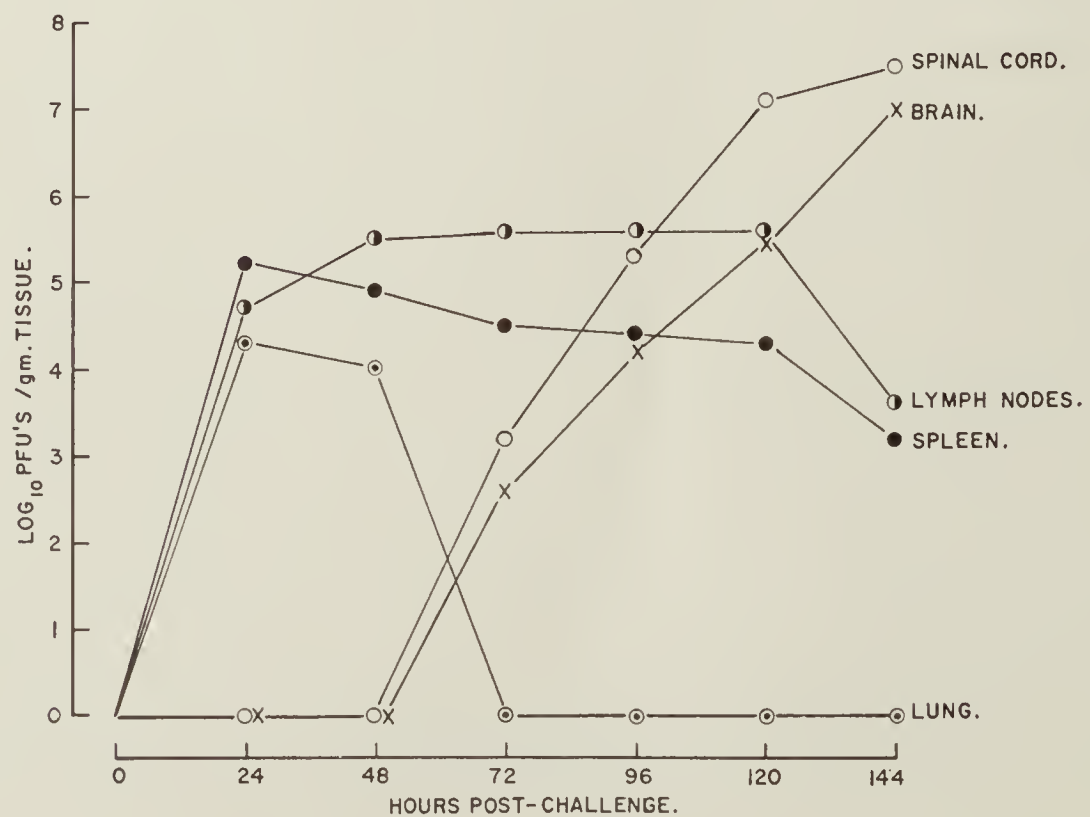


Figure 3.4. The distribution of infectious virus particles in the tissues of mice following the intraperitoneal injection of 10^8 PFU of S-Mengo.

in the spinal cord at 24 hours, and in the brain at 48 hours. The levels of virus in these tissues increased progressively until the death of the mice, with the level in brain lagging behind that in spinal cord. This was true in all experiments in which mice were challenged with a lethal dose of virus. In the experiment depicted in this figure, small amounts of virus were found in kidney, heart, and lung at 24 and 48 hours, but not at later times. Virus particles were not always detectable in these latter three tissues, even in the face of challenges with lethal doses, and even when they were, they were present at low levels. Virus was never detected in either blood or liver.

Figure 3.2 illustrates the distribution of M-Mengo in mice following an intraperitoneal challenge of 50,000 PFU. Virus appeared only in the spleen and lymph nodes, in which tissues it was readily detectable at 24 hours post-challenge. It remained in these tissues at relatively constant levels for some five days, after which it gradually disappeared, until by nine days post-challenge virus was no longer detectable in either tissue. A figure illustrating the distribution of S-Mengo in mouse tissues following the intraperitoneal injection of 50,000 PFU of this variant is not presented here since it was identical in all but unimportant details to Figure 3.2.

From this initial series of experiments, it was evident that the intraperitoneal LD₅₀ for L-Mengo was less than 50,000 PFU, since none of the mice not killed for the collection of tissues survived, and that the intraperitoneal LD₅₀'s for S-

Table 3.1

The Virulence of Mengo Variants in Mice

Variant	Route of Injection	Weight of Mice (g)	LD ₅₀ [*] (PFU)
S	Intraperitoneal	24-26	-†
M	"	"	2-8 x 10 ⁶
L	"	"	50
S	Intraperitoneal	14-16	-†
M	"	"	1-5 x 10 ⁴
L	"	"	1
S	Intracerebral	11-13	5
M	"	"	1
L	"	"	1

*

Calculated by the method of Reed and Muench (1938).

†

Values here are uncertain, but in both cases are probably considerably greater than 10⁷ PFU. See text for details.

and M-Mengo were considerably greater than 50,000 PFU since there were no deaths among those animals not killed for the collection of tissues. The experiments were therefore repeated with the M and S variants, using challenge doses that had been established to produce 100% mortality in recipients. The results are illustrated in Figures 3.3 and 3.4.

The distribution patterns observed following the intraperitoneal injection of lethal doses of S- and M-Mengo are remarkably similar to that observed with the L variant. There are, in fact, no significant differences whatsoever with respect to the four key tissues, - spleen, lymph nodes, brain, and spinal cord. With the S variant (Figure 3.4) no virus was detectable at any time in kidney or heart tissue. However, it is unlikely that this observation has any significance. Virus was not always found in these tissues following the administration of lethal doses of the L and M variants, and even when it was, it was present at very low levels.

The virulence, in mice, of the three Mengo variants when administered via the intraperitoneal route was determined. In this study, mice weighing 24-26 g (that is, the same as those used in the investigation concerning the distribution of virus in mouse tissues following intraperitoneal administration), as well as mice in the 14-16 g range were used. The results, expressed as LD₅₀'s in PFU, are summarized in Table 3.1. It should be pointed out that the intraperitoneal LD₅₀'s for the S and M variants varied from experiment to experiment, although replicate titrations of the L variant yielded data of exceptional reproducibility. For this reason,

ranges of LD₅₀'s for M-Mengo are given in Table 3.1. These were established from a number of titrations carried out over a period of one year. Values for S-Mengo have not been included in the table since there is some doubt as to what these actually are. This point is discussed in the next section.

The LD₅₀'s of S-, M-, and L-Mengo when the variants were administered by the intracerebral route are also shown in Table 3.1. From the data, it is clear that the very large differences in LD₅₀'s observed when the intraperitoneal route of injection was employed largely disappeared when the intracerebral route was used. However, despite the fact that the intracerebral LD₅₀'s for the three variants were found to be essentially identical, it was observed that mice which were injected with either the S or the M variant died approximately two days later than did those which received the same number of PFU of the L variant.

Heterogeneity of S-Mengo

Colter et al. (1965) gave values of $10-35 \times 10^6$ and $1-5 \times 10^4$ PFU of S-Mengo as the LD₅₀'s for 24-26 g and 14-16 g mice respectively, but later work has shown that the S-Mengo pools used in these experiments contained varying amounts (around 1%) of a fourth variant. This variant, which has been designated SL-Mengo, formed minute plaques, almost indistinguishable from those of S-Mengo, under agar overlay, but differed markedly from S-Mengo with respect to its virulence to mice. The intraperitoneal LD₅₀ of the SL variant in 14-16 g mice was found to be 10 PFU. The isolation and some

properties of this variant are described in Chapter 6.

S-Mengo appears to mutate spontaneously to SL-Mengo, since attempts to obtain a pool of the former completely free of the latter by repeated cloning of viral progeny from single plaques failed. It was possible, however, to obtain pools of S-Mengo containing about 0.1% or less of SL-Mengo. Virus from these pools often failed to kill mice even at intraperitoneal doses of 50 million PFU. Of a group of fifteen 14-16 g mice, injected intraperitoneally with 2×10^6 PFU of S-Mengo, two became moribund on the 8th and 9th days. The brains of these mice were removed, homogenized in virus diluent, centrifuged, and the supernatants were assayed for virus. The SL variant was found in high titer, and although no S-Mengo plaques were detected, it is difficult to state categorically that absolutely none of this variant was present.

Adsorption of the variants to tissue homogenates

The studies of the distribution of the Mengo variants in mice following intraperitoneal injection demonstrated that virus was recovered in high titer from spleen and lymph nodes, and in fatal infections, from spinal cord and brain as well. Virus was not found in blood or liver, and only low titers were ever recovered from kidney, heart, and lungs.

Poliovirus and other enteroviruses exhibit well-defined tissue tropisms (Holland, 1961). The possibility was considered then, that Mengo virus was not found associated with kidney, heart, lungs, and liver, due to a lack of receptor material in these tissues. A series of experiments were carried out to test this proposition. Between 6 and 10 young adult

Table 3.2

Adsorption of Mengo Virus by Mouse and Chicken Tissue Homogenates

Species	Tissue	% Unadsorbed Virus					
		S-Mengo Expt. 1	S-Mengo Expt. 2	M-Mengo Expt. 1	M-Mengo Expt. 2	L-Mengo Expt. 1	L-Mengo Expt. 2
Mouse	Brain	52	40	6	3	87	60
	Spleen	20	20	22	19	84	75
	Liver	19	21	16	1	100	108
	Heart	5	9	17	19	102	109
	Kidney	9	21	11	11	99	86
Chicken	Brain	50	33	61	55	96	72
	Liver	64	46	33	29	93	102
	Heart	30	24	40	23	112	102
	Kidney	37	16	80	60	101	100

(24-26 g) mice were exsanguinated and their brains, spleens, hearts, and kidneys removed into cold virus diluent. The tissues were immediately homogenized at 0° in virus diluent with a Potter-Elvehjem grinder fitted with a Teflon shaft. Ten per cent suspensions of these homogenates were then incubated for 30 minutes at 25° with 5-10,000 PFU/ml of S-, M-, or L-Mengo. At the end of the incubation period, the samples were centrifuged at low speed to remove particulate material, and the supernatants were titrated for virus content (that is, for unadsorbed virus). Data from two experiments are shown in Table 3.2. It is quite clear that the virulence of L-Mengo cannot be explained on the basis of a high affinity for mouse tissues in vivo, since with every tissue examined, this variant attached less efficiently than did either S- or M-Mengo. It is also evident that the failure to detect anything but very low levels of S- and M-Mengo in heart, kidney, and liver tissues cannot be explained on the grounds that these tissues lack receptor sites for the agents.

Table 3.2 also shows the recovery of virus following incubation with tissues from two five week old chickens under the same conditions as were used with the mouse tissues. Both S- and M-Mengo were adsorbed to these tissues, although always to a lesser extent than to the corresponding mouse tissue.

In a separate series of experiments, the adsorption of virus to homogenates of the brains of young mice (14-16 g) was examined, and the results are summarized in Table 3.3. With each variant, less virus was recovered than in the experiments with 24-26 g mice (Table 3.2), indicating that the brains of

Table 3.3

Adsorption of Mengo Virus by Young Mouse Brain Homogenates

Variant	% Unadsorbed Virus	
	Expt. 1	Expt. 2
S	16	15
M	7	3
L	37	34

young mice contain more Mengo-adsorbing material than those of older ones. This observation may explain, at least in part, why the intraperitoneal LD₅₀'s of the three variants were found to be considerably lower in 14-16 g mice than in those weighing 24-26 g. (Table 3.1). In this connection, Kunin (1962) has noted a progressive loss, with increasing age, of the adsorptive capacity of mouse brain for Coxsackie B1 virus.

Fate of Mengo virus in blood and peritoneal fluid

The throats of 14-16 g mice were cut and the blood was collected into heparinized virus diluent. The cells (red and white) were spun down and resuspended in growth medium without calcium at a concentration of 6×10^6 cells/ml. The contents of the peritoneal cavities were flushed out with PBS, and the cells were spun down and resuspended in growth medium without calcium at a concentration of 1×10^6 cells/ml. To 3 ml aliquots of these cell suspensions was added 0.3 ml of S-, M-, or L-Mengo at concentrations of 3-5,000 PFU/ml. The suspensions were agitated with small magnetic stirrers and were incubated

at 37°. Controls containing the same amount of virus in growth medium alone were incubated under the same conditions. After 24 hours incubation, all samples were frozen and thawed twice, particulate material was removed by centrifugation, and the supernatants were assayed for virus content. The suspensions of blood and peritoneal cells contained little or no virus, from which it is clear that under these conditions at least, these cells cannot support the replication of Mengo virus.

It was further shown that virus was not adsorbed to any significant extent by washed blood cells. Blood from several mice was collected into citrated medium, and the cells, after one washing in virus diluent, were resuspended in virus diluent at a concentration of 10^7 cells/ml. After incubation at 25° for 30 minutes with about 10^4 PFU/ml of S-, M-, or L-Mengo, the cells were sedimented by centrifugation and the supernatants titrated for virus. Results of one experiment are shown in Table 3.4. In view of the fact that M-Mengo has been shown to have the greatest hemagglutinating activity of the three variants when tested with human group 0 and sheep erythrocytes (Ellem and Colter, 1961), it is interesting that M-Mengo was also adsorbed to the greatest extent by mouse cells.

Table 3.4

Adsorption of Mengo Virus by Mouse Blood Cells

Variant	S	M	L
% Unadsorbed virus	114	51	91

Neither the hemagglutinating ability nor the degree of adsorption of M-Mengo, however, was very marked.

Susceptibility to, and production of interferon by the Mengo variants

One of the most recent hypotheses which has been advanced to explain animal virus virulence involves the production, or lack of production, of interferon. Enders (1960) proposed that it might be generally found that virulent viruses produce less interferon than do avirulent ones, and support for this concept has come from the work of several investigators. For example, Wagner et al. (1963), from studies of large and small plaque variants of vesicular stomatitis virus, concluded that the latter was more sensitive than the former to the action of an exogenous interferon. Sellers (1964) has reported that an avirulent strain of foot and mouth disease virus induced greater yields of interferon in cultures of bovine tongue epithelium than did virulent strains. In a series of two papers, Ruiz-Gomez and Isaacs (1963a,b) have presented evidence to support the proposition that, as a general rule, virulent viruses are poor producers of interferon, and are relatively insensitive to its antiviral action. The possibilities that the high virulence of L-Mengo compared with that of M- or S-Mengo reflected either its relative insensitivity to exogenous interferon, or its relative inefficiency in stimulating interferon production were therefore examined.

Preliminary attempts to demonstrate a direct production of interferon by Mengo-infected L cells failed. Some sublines of L cells, however, are much poorer producers of interferon

than others (Lockart, 1965) and this may explain the failure of these experiments. The logical extension of this work, i.e. to use primary mouse embryo fibroblasts in place of L cells, has not been done. Instead, the effect of exogenous interferon on plaque production of the variants in L cells was investigated.

L cell monolayers were incubated overnight with 2 ml aliquots of a 1:10 dilution in growth medium of the Chikungunya induced interferon preparation described in Materials and Methods. The treated monolayers were then drained, challenged with approximately 60 PFU of S-, M-, or L-Mengo, or VSV, and overlaid one hour later. Although this concentration of interferon reduced the plaque counts of the VSV-infected plates to 10% of the control values, those of the Mengo-infected plates were not reduced at all.

This observation suggested that the Mengo variants were much more resistant to inhibition by interferon than was the highly sensitive VSV, and that the rather crude plaque reduction method could not detect this degree of inhibition. The effect of interferon on total virus production rather than on plaque production was therefore examined as a potentially more sensitive method of detection.

Threefold dilutions of an initial 1:2 dilution of Chikungunya induced interferon were prepared in growth medium, and 2 ml aliquots were incubated with L cell monolayers at 37° for 3 hours. Another set of monolayers was incubated in the same manner with mock interferon or with fresh growth medium. Two ml of growth medium containing a total of about 1000 PFU of S-,

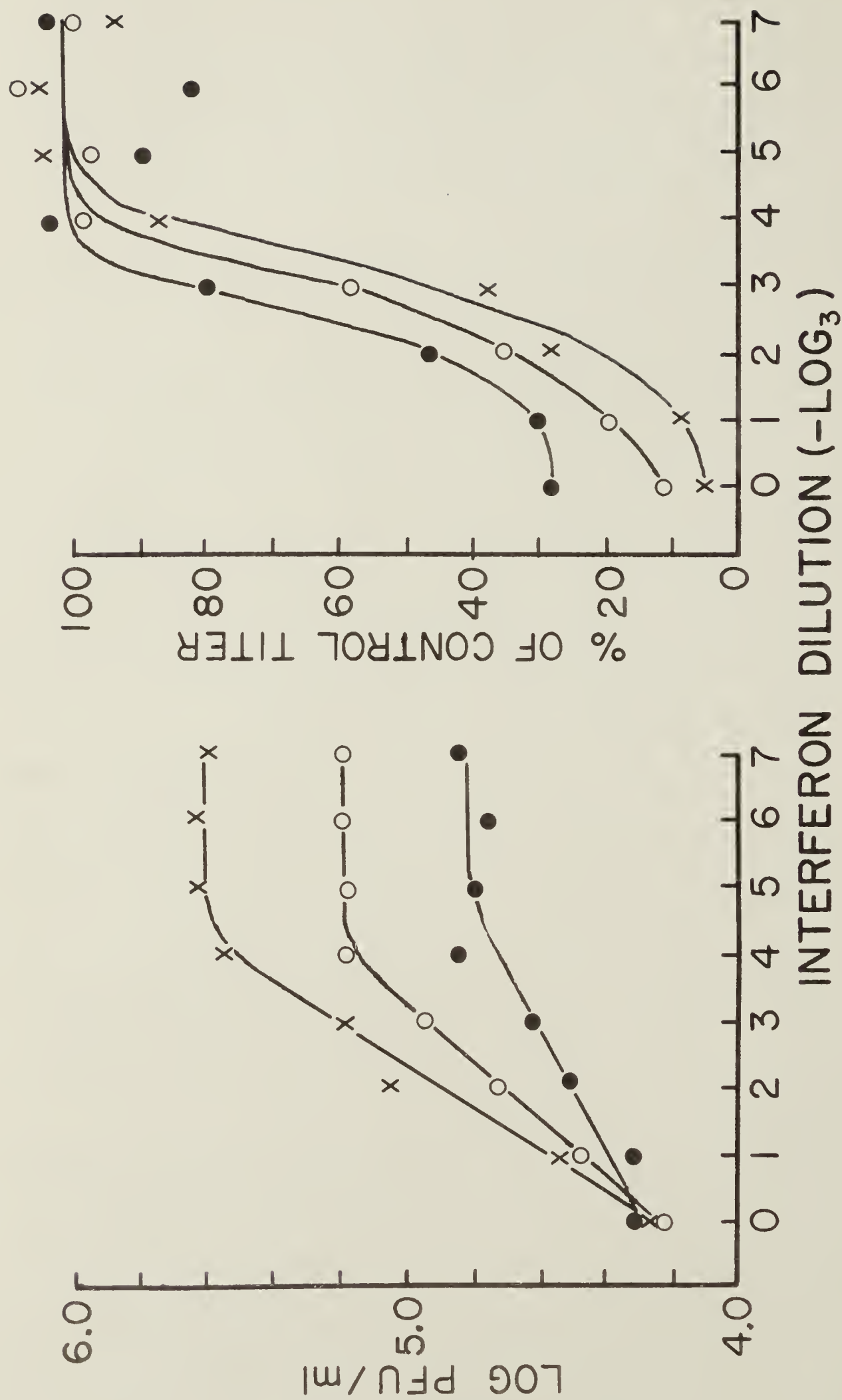


Figure 3.5. Effect of interferon on Mengo virus growth in L cells. Interferon dilutions are expressed as the negative \log_3 of an initial 1:2 dilution of stock material. A. Results presented as absolute titers of virus. In order to facilitate comparison of the variants, different scales have been used: L x 1; M x 40; S x 10. B. The same results expressed as percentages of the titers of control fluids. x = L-Mengo; o = M-Mengo; ● = S-Mengo.

M-, or L-Mengo was then added to each monolayer, and incubation was continued at 37°. Twenty-four hours later, the monolayers were frozen and thawed twice, and the media were titrated for virus content. The results are shown in Figure 3.5. The mock interferon controls contained the same amount of virus as the controls incubated with growth medium alone, indicating that the reduction in titer observed in the presence of interferon was not due to some toxic material from the mouse embryo cells alone. Contrary to hopes and expectations, however, the multiplication of L-Mengo appeared to be most strongly reduced by interferon, and S-Mengo the least affected. In another experiment of this type, M-Mengo was found to be least sensitive to inhibition by interferon, but again multiplication of L-Mengo was the most strongly reduced.

In vivo studies of interferon production by the three Mengo variants have not progressed beyond a preliminary stage, but evidence has been obtained that L-Mengo induces the production of considerably larger amounts of interferon than does S-Mengo. Briefly, these experiments have been of the following format. Thirty to fifty mice (14-16 g) were injected intraperitoneally with a predetermined amount of virus. At daily intervals thereafter, 4-8 mice were killed by exsanguination, and blood, spleen, liver, kidney, lung, heart, and brain collected. The tissues were weighed, homogenized as 10% or 20% suspensions in virus diluent, and were centrifuged to remove particulate material. Small samples of the supernatants were then taken for determinations of virus content. The remaining portions were dialysed at pH 2 to inactivate virus,

and were then titrated for interferon by the plaque reduction method, as described in the Materials and Methods section.

Injection of 2×10^6 PFU of S-Mengo resulted in the appearance in the blood of a small amount of interferon which reached a maximum on the 4th day. Only a very slight interferon-like activity was detected in the brain over the 5 days during which tissues were collected. No mice died or became visibly infected. Injection of 2×10^6 PFU of L-Mengo resulted in a high production of interferon by 24 hours in the three organs so far tested--blood, brain, and spleen. This production declined over the next two days, all mice being dead or dying by the third day. The virus content of the tissues followed the pattern depicted in Figure 3.1, although in this experiment virus was also recovered from the blood. Titers in blood, however, were never high, dropping from a maximum of $10^{3.5}$ PFU/ml on day 1, to $10^{2.8}$ on day 2, and $10^{1.7}$ on day 3. A similar experiment, using sublethal doses of L-Mengo (0.5 PFU) resulted in the production of a low level of interferon, a level of the same order of magnitude as that produced in response to challenge with 2×10^6 PFU of S-Mengo.

Discussion

The data presented in the first section of this chapter make it clear that the distribution of S-, M-, and L-Mengo in the tissues of mice following intraperitoneal injection of the agents is precisely the same--when lethal doses are administered. An examination of the patterns obtained (particularly, perhaps, of those observed in the face of challenges with

sublethal levels of virus) suggests that the primary targets for these agents are the spleen and lymph nodes. Death ensues only when the agents invade the nervous system. The LD₅₀'s of the variants show that L-Mengo can readily effect this invasion; M-Mengo accomplishes it with difficulty, and S-Mengo may not manage it at all. This implies that some barrier system is present in the mouse which prevents access of M- and S-Mengo to the central nervous system but through which L-Mengo can pass with ease. Elucidation of the nature of this barrier, then, is the first necessary step in explaining the high virulence of L-Mengo and the relative avirulence of the M and S variants.

A low species specificity of Mengo virus is suggested by the observations that chicken tissues adsorbed significant amounts of M- and S-Mengo (Table 3.2). L-Mengo is not adsorbed to any extent by chicken tissues, but this is understandable in view of the fact that it appears to have only a weak affinity for mouse brain and spleen, and little or none for mouse liver, heart, or kidney.

In view of the fact that S-Mengo does not appear to be able to reach the central nervous system when injected by the intraperitoneal route, even when administered in massive doses, it is interesting that the apparent affinity of this variant for mouse brain tissue is significantly less than its affinity for other tissues tested, including ones in which it does not appear to multiply. The most obvious implication of this observation, i.e. that S-Mengo cannot multiply in nervous tissue, seems to be ruled out by the observation that the LD₅₀

of S-Mengo, when injected intracerebrally, is only 5 PFU. Nevertheless, the possibility exists that it was SL-Mengo, the virulent strain contaminating pools of S-Mengo, and not S-Mengo itself that killed the mice. The intracerebral LD₅₀ of SL-Mengo is less than 10 PFU, and two experiments using "purified" S-Mengo gave intracerebral LD₅₀'s of the order of 100-1000 PFU. At the time, these experiments were rejected as being atypical, since the distribution of deaths among the groups of mice did not correspond well with the amount of virus injected. It is clear that this point should be reinvestigated.

The low species and tissue specificities of the Mengo variants contrasts markedly with those of poliovirus and other enteroviruses. Kunin (1964) and Holland (1964) have discussed the bases for cellular susceptibility to enteroviruses. Holland found a very high correlation between virus tissue tropisms and virus binding by tissue homogenates:--only homogenates of primate brain, spinal cord, and intestinal tissue adsorbed poliovirus. Homogenates of normally insusceptible primate tissues such as kidney, heart, and lung (with the possible exception of liver) failed to bind any significant amount of virus. Furthermore, the non-neurovirulent type 1 LSc strain was not bound by brain, but was still bound by intestinal homogenates. On the other hand, Kunin (1962) was unable to detect any significant difference between the virulent Mahoney and the attenuated LSc strains of type 1 poliovirus with respect to their ability to adsorb to rhesus monkey brain tissue, and although Kunin and Jordan (1961) and Kunin

(1962) agreed that primate central nervous tissue was most active, they found that minces of all rhesus monkey tissues studied inactivated poliovirus. Adsorption of Cocksackie B1 virus, however, was limited to rhesus monkey brain and liver (Kunin, 1962). Studies were also presented to indicate that age-specific susceptibility and mouse virulence of certain Cocksackie viruses were related to the relative abundance of receptor-like substance in host tissues (Kunin, 1962).

It is evident, however, from the studies measuring the uptake of the variants by tissue homogenates (see Table 3.2) that the high virulence of L-Mengo cannot be explained in terms of a high affinity for tissues of the central nervous system. The decreased susceptibility of older mice to infection with all three variants may, however, be correlated with a decreased affinity of the virus particles for the older nervous system tissue (compare Tables 3.2 and 3.3). If the ability of tissue homogenates to bind the variants can be taken to reflect the affinity of the agents for intact cells, then L-Mengo shows the same low affinity for all types of tissues tested as it does for L cells (see Chapter 2). It is also evident that the reason for the failure of the variants to multiply to any extent in liver, heart, and kidney is not connected with a lack of receptor sites in these tissues.

Clearly, a superficial study of the distribution of the virus in mouse tissues is insufficient to define precisely the pathway or pathways of viral infection. Nevertheless, some interesting features have emerged from the present study which are worthy of discussion and some speculation.

Virus was never recovered from blood or liver, and only low titers were ever found in kidney, heart, and lung. These observations suggest that the blood vascular system is not the main route of infection by the variants. In addition, were this the case, virus would probably have been found in the brain prior to the spinal cord, whereas the converse was found.

L-Mengo killed 50% of mice injected intraperitoneally with only 1 PFU, and it is interesting to speculate on the fate of this single infectious particle. It is reasonable to expect that its initial site of multiplication would be in the region of the inoculation--the peritoneal cavity. Unquestionably, the first cycle of multiplication must produce progeny virus: an abortive cycle in which no virus was produced would terminate the infection. Cells immediately available to the infectious unit would include those in the mesenteric membrane, and fibroblasts and leukocytes in the peritoneal fluid. Two cycles of multiplication in the space of 16 hours or so could produce 50,000 or more infectious progeny virus, which would be liberated into the peritoneal fluid. It is likely that the lymph nodes, exercising their normal function as filters to remove foreign material, would take up this liberated virus. That infectious virus was found in the lymph nodes in the present study is not by itself proof that these organs are capable of supporting virus growth, but in view of the high titers found at 24 hours post-challenge, this is a distinct possibility. Virus from the infected lymph nodes could then pass into the lymph stream and reach the heart and blood stream via the thoracic duct.

Infection of the spleen could arise from blood-borne virus since this organ is interposed not in the lymphatic system but rather in the blood vascular system, although the fact that virus was not also recovered from the liver argues against this possibility. A perhaps more likely route is by direct invasion of the spleen, through the mesenteric membrane or by movement through lymphatic vessels from infected splenic lymph nodes. Once established in the spleen, the virus could gain access to the spinal cord by means of afferent nerves of the autonomic nervous system.

The data presented here do not justify a detailed account of the various theories which have been advanced to explain the neuronal transmission of viruses. It is sufficient to note that this has been a matter of controversy ever since the initial work of Goodpasture and of Marinesco on the development of herpes encephalitis in rabbits following inoculation of a scarified cornea. Both these workers agreed that the mode of transport of virus from eye to brain followed a neural pathway, but Goodpasture favored an ascension through axons of the sensory division of the trigeminal nerve (Goodpasture and Teague, 1923), whereas Marinesco considered that virus was carried centripetally along spaces between fibers of this nerve trunk (Marinesco and Draganesco, 1923). Wright (1953) has given a very clear discussion of these theories in the light of more recent developments, and no more need be said here.

Burnet (1960) has discussed the evidence pointing to the existence of neuronal transmission of a number of neurotropic

viruses, including those of poliomyelitis, rabies, and herpes. Even more pertinent to the present work are reports of the neuronal transmission of murine viruses. Sanders (1953) presented evidence for neuraxonal spread of the GDVII strain of mouse encephalitis virus, which has biological, but not antigenic, characteristics intermediate between Theiler's original strain of mouse encephalitis and EMC virus (Andrewes, 1964). There is little evidence available to support the theory of axonal travel by EMC virus (Sanders, 1953). Neural spread is still implicated, however, since the primary site of multiplication of EMC virus injected intramuscularly appears to be the supporting, non-neural elements of peripheral nerve (McLaren and Sanders, 1959). There is, therefore, considerable precedent for suggesting that Mengo virus invades the central nervous system by neuronal transmission.

Although the study of the distribution of the Mengo variants in mouse tissues suggests that the blood stream is not a main route of infection by these agents, it may be imprudent to dismiss this route as being of little importance. In this study, virus was not found in the blood, or at least not in an infectious form, but viremia occurring as early as one day after infection was noted in the experiments on interferon production by L-Mengo. The discrepancy between these results in the two sets of experiments may, however, be due to the fact that younger mice were used in the interferon study (14-16 g instead of 24-26 g). If this is in fact the explanation, the implication is that the increased resistance of the older mice to infection (see Table 3.1) is due to some component

in blood. Possible candidates for this role are the cells of the reticuloendothelial system and leukocytes. Olds et al. (1961) suggested that induced modification of macrophages prior to virus challenge might alter the course of infection. They found that the LD₅₀ in mice inoculated intravenously with Mengo virus was reduced by 1-2.5 logs by previous treatment with a variety of agents known to be phagocytosed by the reticuloendothelial system. This suggests that under normal conditions, macrophages remove Mengo from the blood stream and that no multiplication proceeds within it. Studies presented here indicate that Mengo virus does not multiply in leukocytes, but the possibility that they do should be reinvestigated in a more rigorous manner. For example, opsonins may be necessary for the phagocytosis of virus particles, and the use of washed blood and peritoneal cell preparations would have eliminated these from the system. In the case of another Columbia-SK virus, the MM strain, Nelson et al. (1964) have reported the occurrence of crystalline arrays of virus particles within the cytoplasm of polymorphonuclear leukocytes in areas of encephalitis in the brains of infected mice. Force and Stewart (1964) have detected Columbia-SK virus in the circulating leukocytes of infected mice, but whether virus was replicating within these cells or was simply being transported was not established.

An intriguing observation has been made concerning the uptake of poliovirus by mouse macrophages. Poliovirus type 1, unlike types 2 and 3, is not cleared from the blood when injected intravenously into mice (Mims, 1964). This appears to be an unusual instance of failure to clear a foreign particle,

and in the absence of any experimental data which would explain it, Mims has attributed it to a shortage of opsonins (Mims, 1964). Blood clearance studies have not been carried out with the Mengo variants, although a preliminary experiment has been done with S-Mengo. Here, 3×10^7 PFU of S-Mengo was injected into the tail veins of four adult mice of 26 g or more. At intervals of 1, 2, 4, and 24 hours thereafter, one mouse was killed, and blood from the jugular vein was examined for virus content. Only a trace of virus was found at 1 hour, and none in the later samples. A comparative study of the blood clearance rates of the three variants in mice after intravenous injection might therefore yield some very interesting information, especially if L-Mengo remained in the circulation for a significantly longer period of time than did M- or S-Mengo. This could be done in conjunction with an examination of the distribution of the variants in mouse tissues following intravenous injection. Such a study could provide very interesting data. Specifically, it should be possible to establish whether L-Mengo is as lethal by this route as by other routes, and whether the distribution of virus in the spleen and liver still follows the same pattern as that observed after intraperitoneal injection.

The importance of macrophages, viremia, and lymphoid tissue in the pathogenesis of virus diseases has been stressed in a recent review which considered the leukocyte and macrophage in terms of their phagocytic activity towards viruses and as possible host cells for viral replication (Mims, 1964). Recently, reports of interferon production by human leukocytes

(Gresser, 1961) and mouse macrophages (Glasgow and Habel, 1963) indicate that these cells may contribute to the host defences in a manner other than by direct virucidal action. Glasgow (1965) has further demonstrated that the addition of leukocytes to primary cultures of mouse embryo fibroblasts infected with vaccinia virus reduces the total virus yield. The inhibition appeared to be due to interferon produced in response to viral infection of the leukocytes. Since L-Mengo, of the three variants, appears to be the most strongly inhibited by interferon, these observations do little to help explain its virulence. They do, however, emphasize the possibility that for L-Mengo at least, the main pathway of infection may involve the nervous system rather than the blood vascular system.

CHAPTER 4

Inhibition of Cell-Virus Interaction by an Agar Factor and by Protamine

Introduction

In the report describing the isolation of S-, M-, and L-Mengo, Ellem and Colter (1961) reported that the substitution of methylcellulose for agar in the plaque assay did not alter the relative sizes of the plaques produced by the three variants, although a strain of EMC virus produced much larger plaques under methylcellulose than under agar overlay. From this, they concluded that although inhibition by an agar component could account for the small size of plaques produced by the EMC virus under agar overlay, the differences in the plaque sizes of the Mengo variants could not be explained on this basis.

In the same year, Takemoto and Liebhaver (1961) published a note in which it was shown that the inhibition imposed by agar on plaque development by a small plaque variant of EMC virus could be reversed by adding polycations such as DEAE dextran or protamine sulfate to the agar overlay. This observation remained untested with the Mengo variants, since the results with the methylcellulose overlay appeared to have eliminated the possibility that agar inhibitors could account for the differences in plaque sizes. Nevertheless, in connection with another series of experiments (to test whether polyamines would inhibit the initial cell-virus interaction) it

was found that the addition of protamine to the agar overlay resulted in the development of plaques of S- and M-Mengo larger than those produced by the L variant.

This chapter summarizes the results of studies arising from this observation, in which it was shown that the sizes of plaques produced by the three variants can be satisfactorily explained on the basis of their differing sensitivities to an agar factor, and that the inhibition imposed by this agar factor can be relieved by the addition of protamine to the agar overlay. In addition, it was found that protamine itself is capable of blocking cell-virus interaction and that the Mengo variants differ in their vulnerability to inhibition by this material. In addition to the results of these investigations, data pertaining to the mechanisms whereby the agar factor and protamine inhibit cell-virus interaction are presented.

Materials and Methods

Agar extract. Regular agar overlay was poured into plastic centrifuge tubes and allowed to solidify. The tubes were then left at 4° for 24 hours and, after the gel was broken up with a spatula, were centrifuged at 30,000 rpm for 30 minutes in a Spinco Model L ultracentrifuge using a No. 30 rotor. The clear supernatant--the agar extract--was carefully removed, sterilized by filtration through a Seitz filter, and stored at -20°.

Agar factor. Virus-inhibiting material was isolated from Noble agar by a procedure similar to that outlined by Agol and Chumakova (1963). It consisted, very simply, of

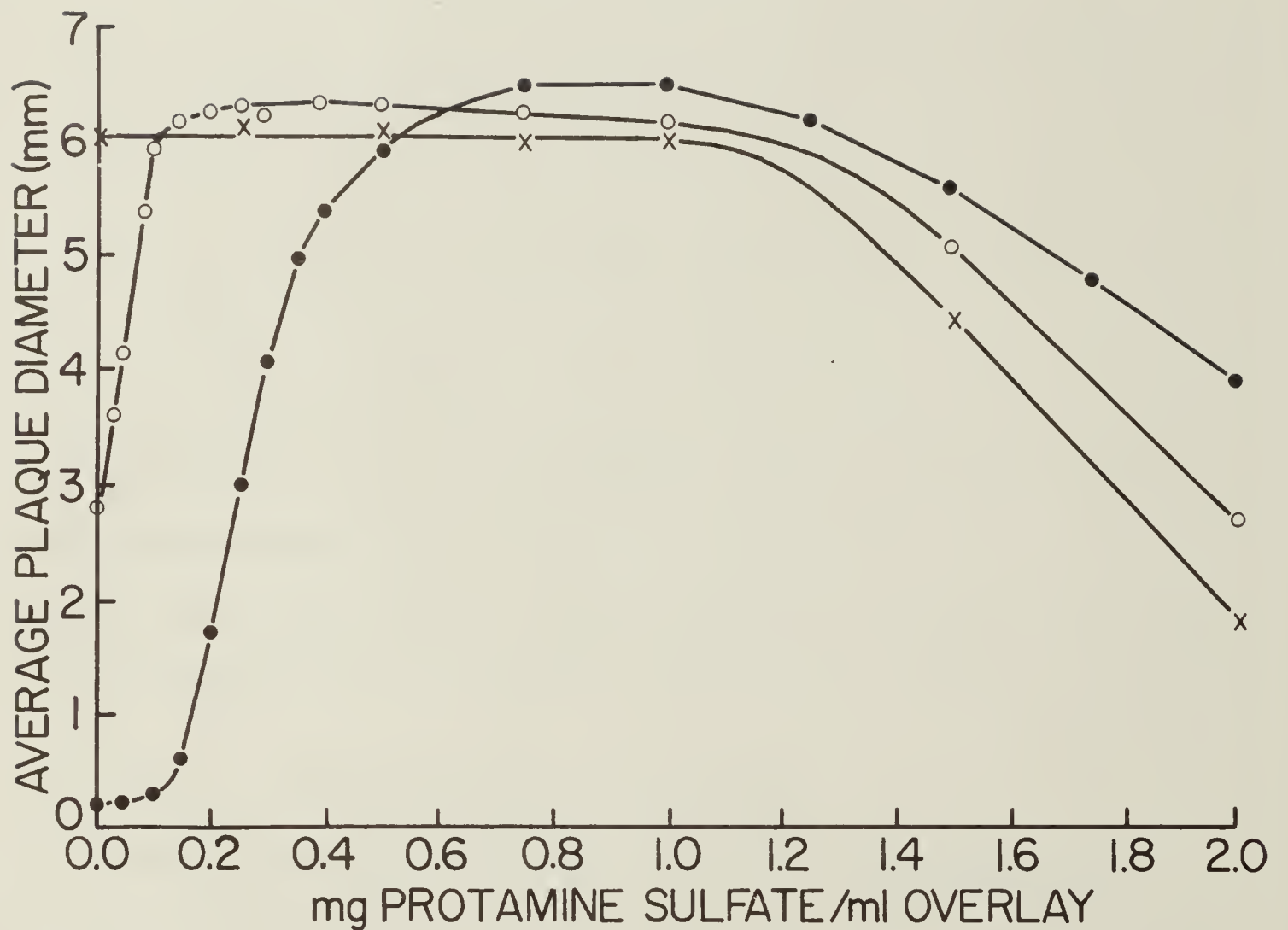


Figure 4.1. The effect of the concentration of protamine sulfate in the agar overlay on the size of the plaques produced by S-, M-, and L-Mengo in L cell monolayers. Plaque diameters were measured after 72 hours' incubation at 37°, and each point is the average of 100 or more individual plaque measurements. ● = S-Mengo; ○ = M-Mengo; x = L-Mengo.

extracting the agar overnight with molar sodium chloride, decanting the supernatant, filtering it carefully through Whatman's Qualitative and then No. 1 filter paper, and precipitating the factor from the clarified extract by the addition of 2 volumes of ethanol. The product, after drying with ethanol and ether, was a faintly brown, finely divided powder which was sparingly soluble in water. It was made up as a 250 $\mu\text{g/ml}$ solution in virus diluent, sterilized by filtration through a Seitz filter, and stored at -20° .

Results

Effect on plaque size of addition of protamine to agar overlay

The fact that an agar inhibitor is an important determinant of the size of plaques produced by the Mengo variants became evident from experiments in which protamine was added to the agar overlay. Under these conditions, it was observed that the S variant, which under normal agar overlay produces plaques of diameter 0.1-0.2 mm, produced plaques at least as large as those produced by the L variant. This prompted a careful examination of the effect of the concentration of protamine in the agar overlay on the sizes of the plaques produced by all three variants.

The results of these experiments are summarized in Figure 4.1. It is clear that the differences in plaque sizes may be eliminated by the addition of the appropriate concentration of protamine. At a protamine concentration of the order of 400-500 $\mu\text{g/ml}$, all three variants produce plaques of essentially the same diameter. Although the size of plaques

produced by the L variant is unaffected by the addition of protamine to the agar overlay, the sizes of those produced by the M and S variants exhibit definite--and quantitatively different--dependencies on protamine concentration. This difference suggested a difference in the sensitivities of the two variants to inhibition by the agar factor, --a proposition substantiated by subsequent studies.

All three variants produced plaques of substantially reduced size in the presence of high concentrations (greater than 1 mg/ml) of protamine in the agar overlay, an observation that is explicable in the light of data presented in a subsequent section. The presence of protamine in the agar overlay at concentrations of 0-1000 μ g/ml, affected plaque size only (plaque numbers were unaffected). However, at concentrations greater than 1000 μ g/ml, a reduction in the number of plaques produced was also noted.

Inhibition of cell-virus interaction by agar extract

Initial studies designed to quantitate the relative sensitivities of the three variants to inhibition by the agar factor were carried out with agar extract in the suspended cell system. The number of infectious centers formed in cell-virus suspensions during an incubation period of 30 minutes at 25⁰ was measured. In this set of experiments, the controls consisted of cell-virus mixtures suspended in a medium of composition identical with that of regular agar overlay except that the agar was omitted. Dilutions of agar extract were made in this medium. With all three variants, a cell concentration of 200,000/ml was employed, and with the M and S

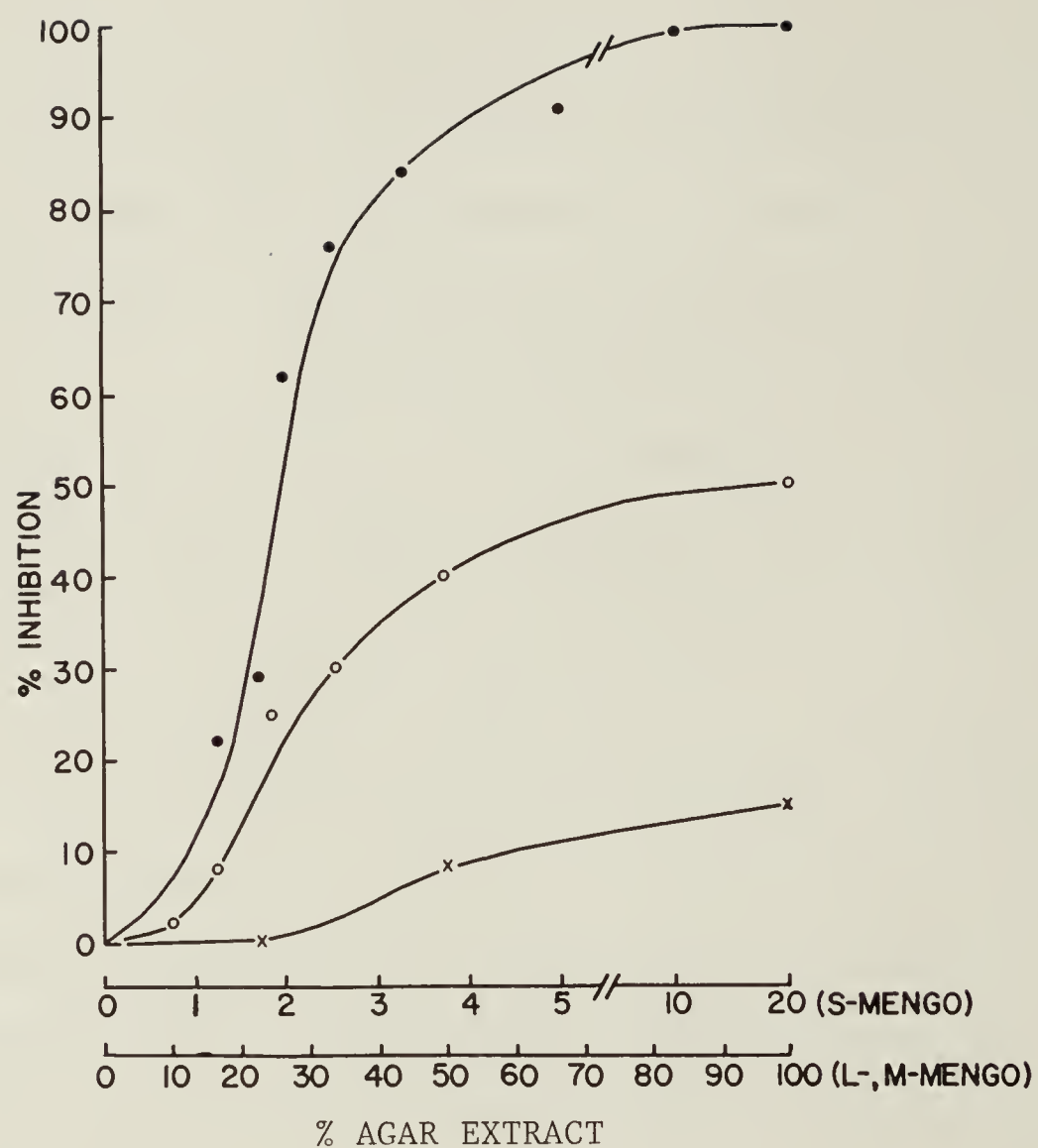


Figure 4.2. Inhibition of L cell--Mengo virus interaction in suspension by agar extract. The incubation mixtures contained 200,000 cells/ml and input virus multiplicities of 5 (S and M) or 10 (L). ● = S-Mengo; o = M-Mengo; x = L-Mengo.

variants, an input virus multiplicity of 5 was used. Under these conditions, 30-40% of the cells in the control suspension became infected and registered as infectious centers. In order to get a significant number (10-20%) of the cells in the L-Mengo system infected, it was necessary to use an input virus multiplicity of 10, reflecting the fact that this variant attaches to L cells much less efficiently than does either the M or S variant.

The results of these experiments are summarized in Figure 4.2. The formation of infectious centers in the S-Mengo system was inhibited to the extent of 50% in the presence of a 1:50 dilution of the agar extract, while 50% inhibition in the M-Mengo system was achieved only in undiluted agar extract. L-Mengo, as expected, was found to be virtually insensitive. Even in undiluted agar extract, an inhibition of only 15% in infectious center formation was observed with this variant.

Inhibition of cell-virus interaction by agar factor and by protamine sulfate

The experimental design employed in these investigations was identical to that outlined in the preceding section except that the suspending medium was virus diluent, pH 7.6, either free of inhibitors (controls), or to which agar factor or protamine sulfate had been added to the desired concentration. Data arising from these studies are summarized in Figures 4.3 and 4.4, in which the percentage inhibition of formation of infectious centers is plotted against the concentration, in the incubation mixture, of agar factor and protamine respectively.

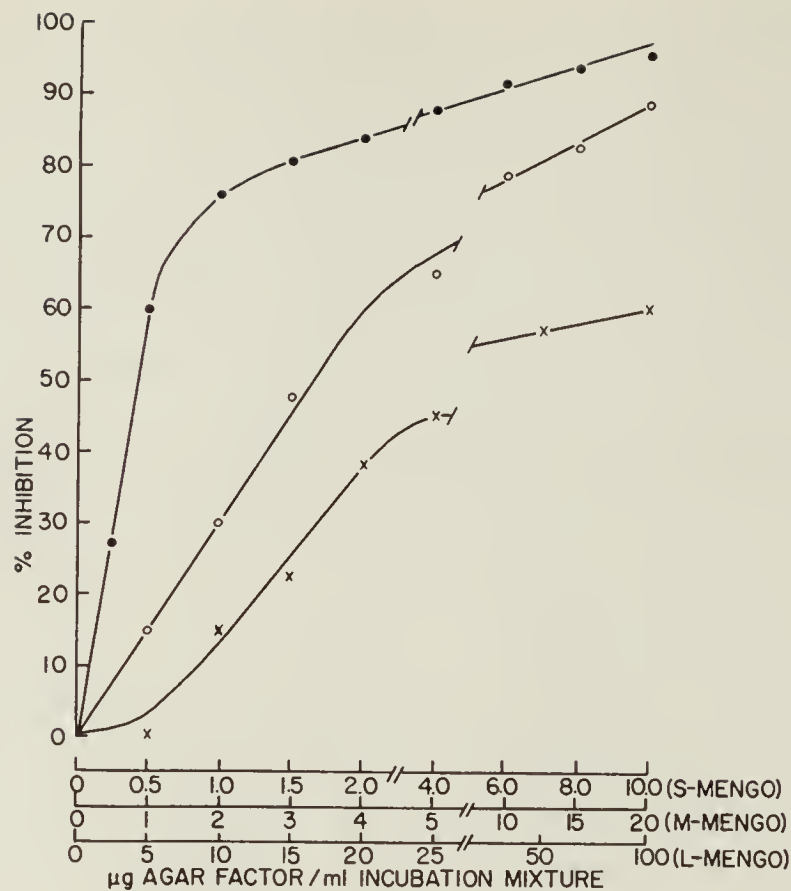


Figure 4.3. Inhibition of L cell--Mengo virus interaction in suspension by agar factor. The incubation mixtures contained 200,000 cells/ml and input virus multiplicities of 5 (S and M) or 10 (L). ● = S-Mengo; o = M-Mengo; x = L-Mengo.

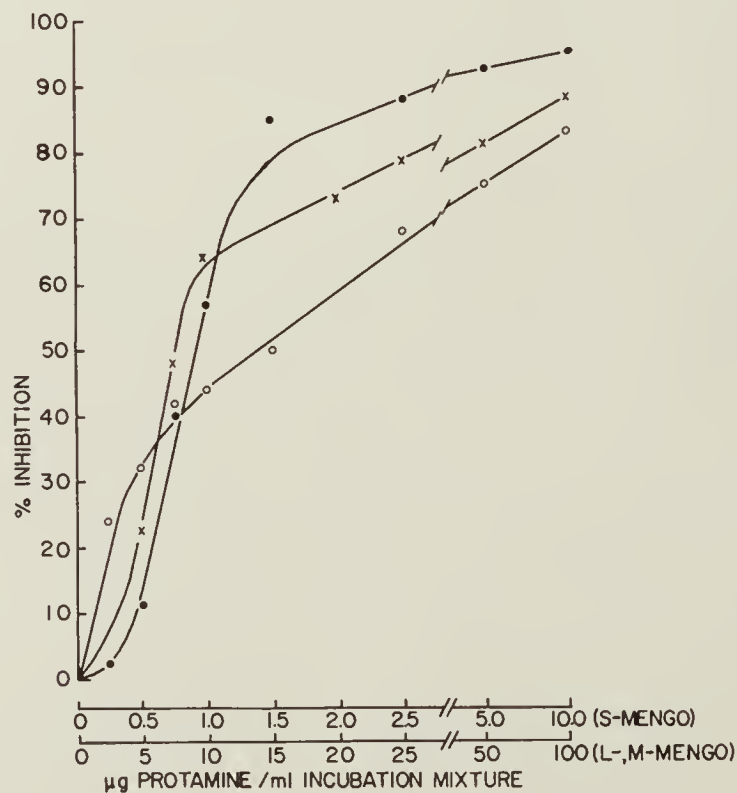


Figure 4.4. Inhibition of L cell--Mengo virus interaction in suspension by protamine. Incubation mixtures contained 200,000 cells/ml and input virus multiplicities of 5 (S and M) or 10 (L). ● = S-Mengo; o = M-Mengo; x = L-Mengo.

The concentrations of these two inhibitors which produced 50% inhibition of infectious center formation with each of the variants are listed in Table 4.1.

Table 4.1

Inhibition of Infectious Center Formation in
Mengo--L cell Mixtures by Agar Factor and by Protamine

Variant	Concentration of inhibitor producing 50% inhibition	
	Agar factor ($\mu\text{g/ml}$)	Protamine ($\mu\text{g/ml}$)
S	0.4	0.9
M	3.3	14.0
L	35.0	7.5

The differences between the variants with respect to their sensitivity to inhibition by the agar factor are quite striking. Expressing their relative vulnerabilities in terms of the concentrations of agar factor that inhibit cell-virus interaction by 50%, one may say, as an approximation, that the S variant is 10 times as sensitive as the M variant, and the M variant 10 times as sensitive as the L.

It was somewhat surprising to find that protamine, which when added to agar overlay, reverses the inhibition imposed by the agar factor, is itself a relatively efficient inhibitor of cell-virus interaction. As was the case with the agar factor, the S variant was found to be the most sensitive of the three variants to this basic protein, less than 1 $\mu\text{g/ml}$ producing a

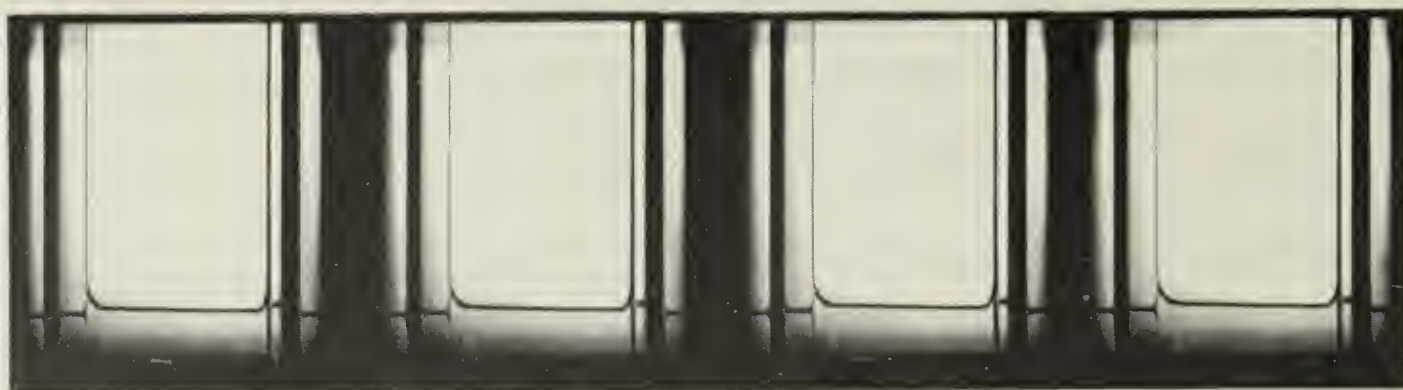


Figure 4.5(i). Representative schlieren patterns of the agar factor taken at 16 minute intervals in a Spinco model E ultracentrifuge during the approach to sedimentation equilibrium. Bar angle = 75° ; speed = 12,590 RPM; temperature = 20° ; $c = 0.5\%$ agar factor in 0.02M phosphate, pH 7.4. Calculations (at the meniscus only) of molecular weight gave a mean value of 50,940, assuming a value of 0.622 ml/g for the partial specific volume, \bar{v} .

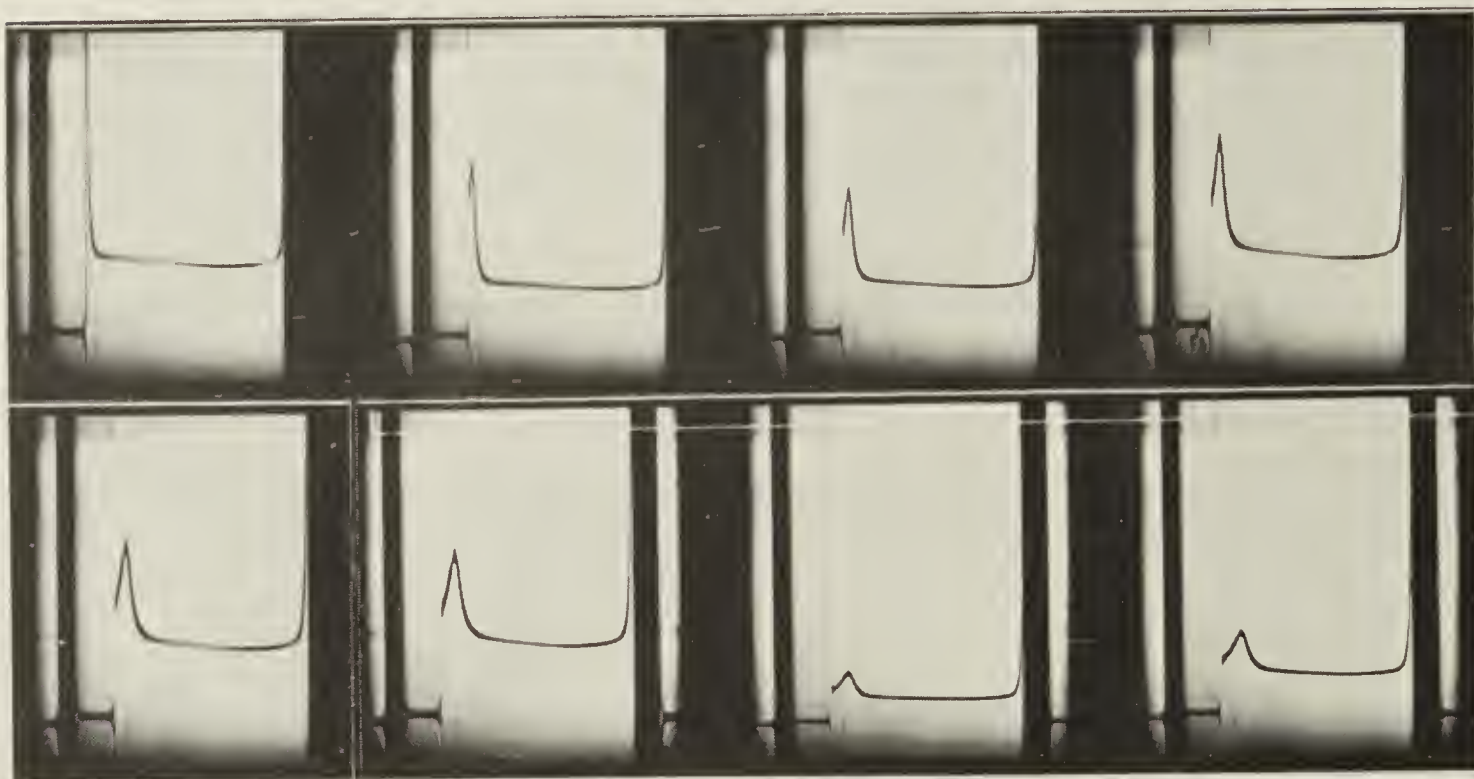


Figure 4.5(ii). Sedimentation velocity patterns of agar factor taken at intervals of 8 minutes, at a speed of 59,780 RPM. Bar angle varied from 50° to 75° ; temperature = 20° ; $c = 0.5\%$ agar factor in 0.02M phosphate, pH 7.4. Assuming a value of 0.622 ml/g for \bar{v} , the $S_{20,w} = 1.48$ S. This is lower than would be expected for a molecule of molecular weight 50,000, and may reflect an extreme asymmetry of the particle due to a high density of net negative charge.

50% inhibition in its interaction with L cells. Unlike the situation with the agar factor, however, the L variant is at least as vulnerable to inhibition by protamine as is the M variant.

Characterization of the agar factor

A study of the mechanism by which virus-cell interaction is inhibited by the agar factor was hindered by lack of knowledge concerning its structure. An attempt was made, therefore, to define its structure especially with regard to its molecular weight and to any charged groups it might contain. A preliminary examination verified that it was a sulfated polysaccharide, as found by Takemoto and Liebhaber (1961), Schulze and Schlesinger (1963b), and Agol and Chumakova (1963) with similar extracts of agar.

Ultracentrifugal studies showed that the preparation was monodisperse, having a molecular weight of about 50,000 (Figure 4.5). Chromatography of acid hydrolysates (2M HCl; 12 hours at 100°) using two solvent systems (EtOAc: HOAc: H₂O (3:1:3), and i-PrOH: H₂O (4:1)) indicated that the major component was galactose. The hydrolysates gave a negative reaction with ninhydrin. Estimation of uronic acid by the method of Dische as modified by Bitter and Ewins (1961) and of phosphate by the method of Fiske and Subbarow (1925) indicated that neither was present. Hydrolysis in 0.15 M H₂SO₄ at 100° resulted in the formation of a compound which strongly absorbed ultraviolet light, with a maximum absorption at 285 mμ. Galactose, at a similar concentration and hydrolysed under the same conditions, gave very little absorbance at this wavelength. It seems

probable that this ultraviolet-absorbing material was 5-hydroxymethyl-2-furaldehyde and that it arose from the breakdown of 3,6-anhydrogalactose (Moye, 1964; Smith et al., 1955). These tentative conclusions are supported by the fact that agarose and agaropectin, the main constituents of agar, consist mainly of galactose and 3,6-anhydrogalactose (Araki, 1959). Using the method of Smith et al. (1955), the increase in u.v. absorption of a 0.15 M H₂SO₄ hydrolysate of the agar factor was studied over a period of 24 hours. By this method, and accepting the above tentative conclusions, it was estimated that approximately one-third to one-half of the weight of the agar factor may be accounted for as 3,6-anhydrogalactose.

Microanalyses of saline-extracted material from Noble agar (agar factor) and from Bacto agar were carried out by Dr. Alfred Bernhardt, Mikroanalytisches Laboratorium, Max-Planck-Institut für Kohlenforschung, 433 Mülheim (Ruhr), Germany, and the data obtained are summarized in Table 4.2.

Table 4.2

Chemical Analysis of Inhibitor Preparations Isolated by Saline Extraction of Noble and Bacto Agars

Type of agar	Percent of dry weight							
	C	H	O	N	S	SO ₄	CH ₃ CO*	Ash
Noble	30.99	5.58	41.55	0.29	3.34	3.08	11.60	15.2
Bacto	29.52	5.23	43.94	0.59	3.53	5.22	11.50	15.7

* Volatile acid calculated as CH₃CO.

For the determination of sulfate, samples were hydrolysed in sealed glass ampoules for 12 hours at 100° , and BaSO_4 precipitated by the addition of excess BaCl_2 was weighed. The two preparations were found to contain almost the same amount of sulfur (3.3-3.5%), but the Noble agar factor appeared to contain less sulfate sulfur than did the inhibitor isolated from Bacto agar. These results are not in good agreement with the value of 15-17% given by Agol and Chumakova (1963) for the sulfate content of an apparently very similar saline extract of Bacto agar.

Apart from a difference in the sulfate content, the preparations from the two types of agar appear to have very similar compositions. It is interesting that both contain the same amount of volatile acid: over 11% when calculated as CH_3CO ,--an amount that could be accounted for by the presence, in the agar factor, of one molecule of pyruvate for every two hexose residues. This suggestion is made since it has been shown that agaropectin contains pyruvic acid connected through acetal linkages with carbons 4 and 6 of the D-galactose residues (Hirase, 1957).

Mainly on the basis of indirect evidence, then, and by analogy with analyses of other agar components (Araki, 1959), it is tentatively suggested that the agar factor has a structure similar to that of agaropectin.

Mechanisms of action of agar factor and protamine

A number of studies were carried out in an attempt to obtain information regarding the mechanisms whereby these two materials block cell-virus interaction. It seemed unlikely

that these inhibitors (a sulfated polysaccharide and a basic protein) would exercise their inhibitory capacities in the same manner, or even at the same site in the cell-virus system. It was postulated that the agar factor acts by immobilizing the virus particles, and that the protamine acts by binding to the cell in such a way that the virus particles are denied access to cellular receptor sites. The data presented below support this premise. While these investigations were in progress, Liebhaber and Takemoto (1963), in a report describing the effect of sodium dextran sulfate on plaque development by EMC virus, suggested that sulfated polysaccharides inhibit virus growth by reacting directly with virus growth by reacting directly with virus particles, and Schulze and Schlesinger (1963), and Schulze (1964) published data showing that the naturally occurring agar inhibitor can, in fact, combine directly with dengue-2 virus.

One approach used in the present study was to examine the effect of incubation of L cells with agar inhibitor or with protamine on the susceptibility of the cells to subsequent infection with S-Mengo virus. Aliquots of cells were incubated for 30 minutes at 25⁰ in undiluted agar extract, in medium containing 500 µg protamine/ml, or in medium containing neither inhibitor. The cells were then sedimented by centrifugation, washed twice in normal medium, resuspended in 5 ml of a suspension of S-Mengo (to give a suspension containing 200,000 cells/ml, and a virus multiplicity of 5), and the number of infectious centers produced during an additional 30 minutes incubation measured. The results of two such

experiments are shown in Table 4.3. It is clear that incubation of cells with agar factor had no effect on their susceptibility to subsequent infection by S-Mengo, while cells incubated with protamine exhibited a decreased vulnerability to infection.

Table 4.3

Effect of Exposure to Agar Extract and Protamine on the Susceptibility of L Cells to Subsequent Infection with S-Mengo

Cell sample	% of Cells Infected	
	Expt. #1	Expt. #2
Control	55	51
Incubated in agar extract	51	53
Incubated in protamine solution	26	32

The fact that protamine, but not the agar inhibitor, may bind to L cells was shown in still another way. L cells were suspended in solutions of agar extract and of protamine containing concentrations of inhibitors just adequate to give essentially complete inhibition of L cell--S-Mengo interaction. After incubation for 30 minutes at 25⁰, the suspensions were centrifuged to remove the cells, and the supernatants were used as suspending media in which to measure S-Mengo--L cell interaction. Illustrative data, presented in Table 4.4, show that the inhibitory capacity of media containing protamine was removed by incubation with L cells, while that of media containing the agar inhibitor was very little affected.

Table 4.4

Effect of Incubation with L Cells on the
Inhibitory Capacity of Media Containing
Agar Inhibitor and Protamine

Media	% of cells infected
1. Virus diluent	20.0
2. 1:50 dilution of agar extract in virus diluent	1.8
3. 1:50 dilution of agar extract in virus diluent, adsorbed with L cells	2.7
4. Virus diluent	25.0
5. Virus diluent containing 2 µg protamine/ml	1.6
6. Virus diluent containing 2 µg protamine/ml, adsorbed with L cells	26.0

Evidence supporting the premise that the agar factor reacts directly with the sensitive variants was obtained by the simple expedient of incubating S- and M-Mengo for 60 minutes at 25⁰ with the agar inhibitor and with protamine, and then determining the number of PFU remaining in the mixtures by titration, after dilution, on L cell monolayers. Control samples of virus, incubated for the same period of time and under the same conditions, were included. The terminal dilutions of control samples (that is, those dilutions applied to the monolayers) were made in virus diluent containing the same concentration of agar inhibitor or protamine that was present in the terminal dilutions of the

Table 4.5
Effect of Incubation with Agar Factor and
Protamine on the Infectious Titters of M-
S-Mengo Pools

Virus sample	Titer (PFU/ml)	
	Expt. #1	Expt. #2
S-Mengo + agar factor	2.0×10^7	3.8×10^7
S-Mengo - agar factor control	5.4×10^7	6.3×10^7
S-Mengo + protamine	5.1×10^7	6.6×10^7
S-Mengo - protamine control	5.1×10^7	6.2×10^7
M-Mengo + agar factor	3.7×10^7	8.1×10^7
M-Mengo - agar factor control	10.3×10^7	21.8×10^7
M-Mengo + protamine	9.6×10^7	20.2×10^7
M-Mengo - protamine control	10.8×10^7	18.9×10^7

Mengo variant-agar factor mixtures contained 100 µg agar factor per ml.

Mengo variant-protamine mixtures contained 250 µg protamine per ml.

virus-inhibitor mixtures. These concentrations were much lower than those required to produce any evidence of inhibition in the suspended cell system.

The results of these experiments are documented in Table 4.5, from which it may be seen that incubation of M- or S-Mengo with agar factor resulted in a loss of roughly 50% of detectable PFU. No loss of PFU resulted from the incubation of these variants with protamine sulfate.

Discussion

The literature now contains numerous reports describing the inhibitory properties of agar towards a wide spectrum of viruses. These include strains of poliovirus types 1, 2 and 3 (Takemori and Nomura, 1960; Agol and Chumakova, 1962, 1963; Takemoto and Liebhaver, 1962b; Voss et al., 1964), ECHO 6 (Barron and Karzon, 1965), Coxsackie A9 (Takemoto and Liebhaver, 1962b) and B4 (Choppin and Eggers, 1962), foot and mouth disease virus (Bengtsson et al., 1963), EMC (Takemoto and Liebhaver, 1961; Závadová and Závada, 1965), dengue type 2 (Schulze and Schlesinger, 1963b; Schulze, 1964), viruses of the equine encephalitides (Brown and Packer, 1964; Colón and Idoine, 1964), a number of other arboviruses including Semliki Forest, Murray Valley encephalitis, and St. Louis encephalitis (Miles and Austin, 1963), Newcastle disease virus (Thiry, 1964), influenza A and B (Takemoto and Fabisch, 1963), and herpes simplex virus (Takemoto and Fabisch, 1964). To this list can now be added Mengo encephalomyelitis virus, and the suggestion of Ellem and Colter (1961) that the marked differences in the

sizes of plaques produced by the three variants could not be explained on the basis of an agar inhibitor must be discarded. Data presented herein show that the differences in plaque sizes can be satisfactorily explained by differences in the sensitivities of the three variants to an agar factor.

The fact that protamine, which permits normal plaque development by the S and M variants when added to agar overlay, is itself capable of blocking cell-virus interaction was a somewhat surprising observation. This basic protein almost certainly reverses the inhibition imposed by the agar inhibitor by forming a complex with it--a premise supported by the observation that a precipitate forms when a solution of agar factor and protamine are mixed.

Data presented here strongly support the hypothesis that the inhibiting material from agar blocks cell-virus interaction by reacting directly with the virus particles, most likely at, or near, those sites through which the viruses attach themselves to cells. The present results with the agar factor and agar extract, therefore, are in accord with those of Schulze (1964), and several other groups who have presented evidence for the direct interaction of viruses and other polyanions [e.g. poliovirus and dextran sulfate (Bengtsson, 1965), influenza virus and dextran sulfate (Styk and Rada, 1964), EMC virus and dextran sulfate (Liebhaber and Takemoto, 1963), herpes virus and heparin (Vaheri and Cantell, 1963), influenza virus and polyphloroglucinol phosphate (Penttinen, 1957)],

Although a sulfated agar polysaccharide appears to be taken up by L cells (Takemoto and Liebhaber, 1962a), the data

presented in Tables 4.4 and 4.5 make it improbable that the agar factor inhibits the formation of infectious centers from an intracellular location. The possibility that protamine has an intracellular site of action, however, has not been ruled out completely on the basis of the experiments described here. Protamine has been reported to uncouple oxidative phosphorylation (Rivenbark and Hanson, 1962). From an intracellular site, then, it could conceivably prevent viral synthesis by removing the main energy source, and the result would be a decrease in the number of infectious centers formed. Nevertheless, it is difficult to account for the differing sensitivities of the three variants to inhibition by protamine on the basis of such a theory.

Protamine sulfate has been used fairly extensively in the purification of a large number of viruses, either by precipitating cellular debris or by precipitating the viruses themselves. Viruses which are precipitated by protamine include influenza A (Chamber and Henle, 1941), polyoma (Sheinin, 1962), herpes, LCM, mouse encephalitis (GDVII strain), rabies, and vaccinia (Warren et al., 1949a). Viruses which are not, include WEE, West Nile, St. Louis encephalitis, Japanese encephalitis, polio (Lansing) (Warren et al., 1949a), and most important of all from the viewpoint of the present work, EMC virus (Weil et al., 1952; Kaighn et al., 1964). This is in accord with the data presented in Table 4.5 indicating that the variants of Mengo virus do not complex with protamine.

If, then, the proposed mechanisms are accepted, certain conclusions regarding the nature of the variants and their attachment to L cells almost inevitably follow. If the agar factor inhibits cell-virus interaction by attaching directly to the virus particles, the very fact that the three variants showed such widely divergent sensitivities to the inhibitor implies that the efficiency with which the latter attaches to the virus particles differs sharply from one variant to another. The alternative to this is that the agar factor binds to different sites on the three variants. In either case, and since Mengo virus is a simple nucleoprotein, the implication is that the variants differ from each other with respect to the charge pattern of their external surfaces. Putting this in another way, the data suggest that the different properties of the Mengo variants result from one or more differences in the amino acid composition of the protein subunits which comprise their outer coats. This suggestion is consistent with the conclusions drawn on the basis of the rate of attachment and pH studies presented in Chapter 2.

Although the initial enterovirus-cell interaction is almost certainly electrostatic in nature, little is known about the actual mechanisms involved. The high specificities of some enteroviruses with regard to the types of cells to which they will attach indicate the presence of specific receptor sites on the surfaces of both virus and cell. Cellular receptors, for poliovirus at least, appear to be heat-labile lipoproteins (Holland, 1964). Proteolytic enzymes will selectively inactivate enteroviral receptors on HeLa cells

without affecting cell viability (Zajac and Crowell, 1965a). Treatment of HeLa cells with trypsin destroys the receptor sites for poliovirus T1 although Cocksackie virus B3 still attaches. Conversely, treatment with chymotrypsin removes the receptors for the Cocksackie but not for the polio virus (Zajac and Crowell, 1965b). Two more group B Cocksackie viruses, B1 and B5, were tested, and were found to behave in the same manner as B3 (Zajac and Crowell, 1965b). These results indicate that poliovirus receptors can be distinguished from those of group B Cocksackie viruses. It remains to be seen whether other enteroviruses, and other viruses in general, can be differentiated on such a basis.

Accepting that the Mengo variants differ in their surface charges, it remains to be determined whether these differences involve the actual viral "active centers" or whether only adjacent groups are involved. By the term "active center" is meant the hypothetical grouping of charges, large or small, on the virus surface which is complementary to a grouping on the surface of a susceptible cell. The tidiest explanation is that the three variants have identical "active centers", and that they differ only with respect to the amino acid residues that surround or are adjacent to the active center. If this is in fact the case, treatment of L cells with a selection of proteolytic enzymes should result in the same pattern of loss or retention of cell receptors for each of the three variants. If an enzyme could be found, however, that resulted in the loss of receptor sites for only

one or two of the variants, it would be hard to escape the conclusion that the three variants do differ with respect to their "active centers". This should prove to be an interesting search.

CHAPTER 5

Effect of Overlay and Polyanions on Plaque Size

Introduction

In the previous chapter, it was shown that the widely differing plaque sizes produced by the three Mengo variants under agar overlay could be satisfactorily explained on the basis of their differing sensitivities to a sulfated agar polysaccharide, and that the inhibition imposed by this factor could be relieved by the addition of protamine to the overlay. The present section describes further studies of the effects of agar, and of its constituents, on the sizes of plaques produced by the variants, and presents data arising from an extension of the work with protamine, in which the effects of a number of anionic polymers on Mengo plaque development under regular agar overlay were examined.

Materials and Methods

Added polymers. Concentrated solutions of the following materials were made up in sterile distilled water, and were diluted to three times the desired final concentration in overlay diluent. These solutions, when mixed with two parts of 1.5% Noble agar, gave the desired concentrations for plating:

Dextran sulfate 500, Na salt (Pharmacia, Uppsala, Sweden) .

Dextran sulfate, types 15S, 60S, 200S, 500S, 2000S, and 5-40S, Na salts (Sigma Chem. Co., St. Louis, Mo.).

Sulfopolyglucin, K salt (Riker Laboratories, Northridge, Calif.).

Dextran (Abbott Laboratories, Chicago, Ill.) M.W. 80,000.

Dextran, types 15, 150, and 5-40 (Sigma Chem. Co.).

Heparin, Na salt, 170 or 179 units/mg (Sigma Chem. Co.).

Chondroitin sulfate, Na salt. A preparation of mixed A and B forms, from bovine nasal septa cartilage (Sigma Chem. Co.).

Hyaluronic acid and sulfated derivatives, Ba salts.

Kindly supplied by Dr. D. L. Cook, G. D. Searle & Co., Ltd., Chicago, Ill.

Polyphloroglucinol phosphate, Na salt (Leo 137a),

Polyphlorethin phosphoric acid (Leo 101a),

Dextran with acid esters of phthalic acid (Leo 127-129a).

All the Leo preparations were kindly supplied by Dr.

Hans Fex of Aktiebolaget Leo, Hälsingborg, Sweden.

Protamine sulfate (salmine) Calbiochem, Los Angeles, Cal.

Methylcellulose overlay. Methylcellulose (4000 centipoises; Fisher Scientific Co., Fairlawn, N. J.) was washed repeatedly with 95% ethanol and then ether and air-dried. Suspensions of the washed material, at 1.5 times the required final concentration, were made in deionized water and autoclaved at 125^o for 15 minutes. After cooling, one-half volume of overlay diluent was added, and the mixture was shaken at 4^o in a mechanical shaker to achieve a uniform consistency. It was stored at 4^o and was used within a week of its preparation.

Preparation of agarose. Agarose was prepared by the

method of Araki, as modified by Hjertén (1961). However, as Hjertén (1962) has pointed out, this method gives a somewhat degraded product, and the method was further modified as follows. Dry acetylated agar, prepared from Noble agar, was shaken with chloroform in a separating funnel, as detailed by Hjertén (1961). The chloroform solution was removed, and the remaining insoluble material (acetylated agaropectin) was extracted once again with the same volume of chloroform. The extracts were pooled and filtered carefully with suction through Whatman's Qualitative and then several times through Whatman's No. 1 filter paper. An equal volume of petroleum ether was added slowly with vigorous mechanical stirring, followed by the same amount of 95% ethanol. The precipitate which formed was allowed to settle, and the supernatant was decanted and discarded. More ethanol was added to remove most of the remaining petroleum ether, the precipitate was sucked dry on a Buchner funnel, washed with ethanol and ether, and air-dried. The resulting fine white powder was deacetylated by stirring as a 10% suspension for 2 hours with 1 M ethanolic KOH (approximately 90% ethanol). The deacetylation procedure was stopped by neutralization with glacial acetic acid. It was necessary to be sure of complete neutralization, as even at mildly alkaline pH's the agarose was found to be degraded upon autoclaving. The suspension was then filtered under suction, the precipitate was washed several times with ethanol, and dried with ether and air. The resulting material was a white powder which gave a semi-opaque white gel without the slightly brown tinge of an agar gel. This method of preparation

gives a product having at least as great a gelling capacity as agar of the same concentration. The agarose is obtained in yields of about 50%.

In the later stages of the present work, a commercial agarose preparation was examined. On the basis of a comparison of plaque sizes of the Mengo variants under both agarose preparations (see Table 5.2) it appears that the commercial material contains more unremoved inhibitor and has slightly poorer gelling ability than material prepared as described here.

Plaque measurements. 0.1 ml of virus dilutions, calculated to contain 15-50 plaque-forming units (depending on the plaque size expected) were pipetted onto L cell monolayers. After one hour's incubation at 37° in a humidified atmosphere of 5% CO₂ in air, the small amount of fluid on the monolayers was removed by suction and 4.5 ml of the appropriate overlay was applied. Incubation, under the same conditions, was continued for 72 hours.

It was found that although early staining of the plates did not affect plaque numbers, it did inhibit plaque sizes. For this reason, plates were overlaid with 3 ml of regular agar overlay containing 0.01% neutral red not more than 3-4 hours before the end of the 72 hour incubation period. Plaque diameters of all visible plaques were then recorded. Methylcellulose-overlaid plates were treated with the agar overlay stain after removal of the original overlay.

It was sometimes inconvenient to measure the plaques at the required time, in which case the following device was

employed. After staining, the plates were carefully covered and stored at -20° for 24 hours or longer. They were then allowed to thaw at room temperature for 1 hour, whereupon the semi-solid overlays could be removed easily with the aid of a spatula by sliding them off the inverted dishes, leaving the monolayers and plaques intact. After drying at 37° , the red color of the neutral red could be restored by inverting the dried monolayers over petri dish covers moistened with glacial acetic acid. If concentrated hydrochloric acid was substituted for acetic acid, the monolayers quickly turned a deep blue color which slowly changed to a permanent red. Plaque diameters could then be measured at any convenient time.

Results

Plaque sizes under methylcellulose overlay

In the initial report describing the isolation and plaque morphology of the S, M, and L variants of Mengo virus, Ellem and Colter (1961) concluded that the marked differences in plaque size produced by the three variants could not be explained on the basis of inhibition by an agar factor. This conclusion was based on the observation that the substitution of methylcellulose for agar did not alter the relative sizes of the plaques produced by the variants, whereas a strain of EMC virus, which produced minute plaques under agar, gave plaques of greatly increased size under methylcellulose. Hence it was reasoned that although the minute plaque size of the EMC virus could be explained on the basis of inhibition by agar, that of the S-Mengo variant in particular, could not. Studies

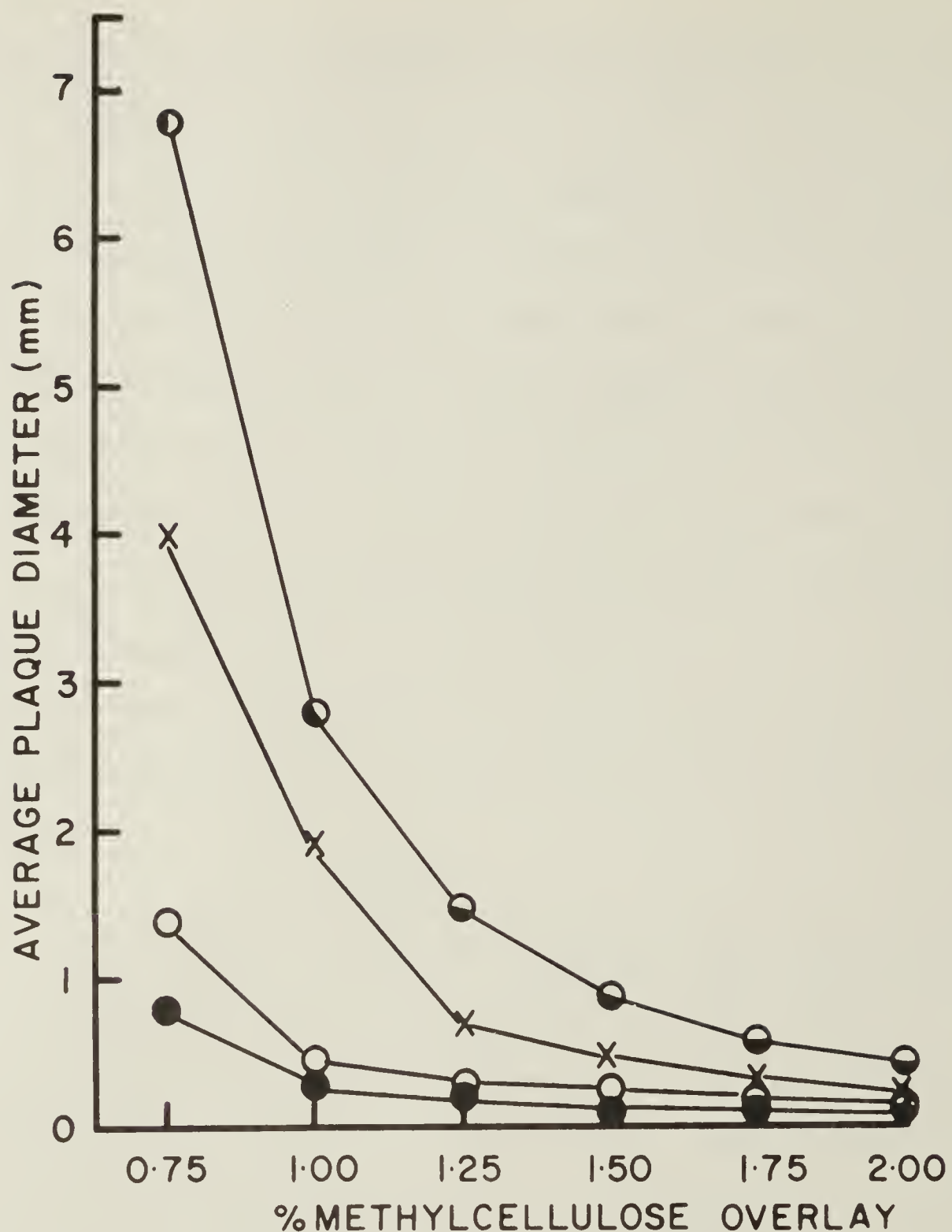


Figure 5.1. Plaque sizes of the Mengo variants and EMC virus under overlays containing varying concentrations of methylcellulose. Plaque diameters were measured after 72 hours' incubation at 37°, and each point is the average of 100 or more individual plaque measurements. ● = S-Mengo; ○ = M-Mengo; x = L-Mengo; ● = EMC.

presented in the previous chapter showed that the difference in plaque sizes of the three variants could, in fact, be satisfactorily explained on the basis of differing sensitivities to inhibition by an agar inhibitor. This observation led to a more detailed investigation of the effect of methylcellulose on the sizes of plaques produced by the Mengo variants and by the minute plaque variant of EMC virus. The results are summarized in Figure 5.1. It is quite clear that S-Mengo produces much smaller plaques under all concentrations of methylcellulose tested than does EMC virus, although both viruses produce plaques of similar size under regular agar overlay. As with S-Mengo, the addition of protamine to regular agar overlay results in a large increase in EMC plaque size (Table 5.1) showing that plaque development by both viruses

Table 5.1

Effect of Protamine Sulfate Concentration in Regular
Agar Overlay on EMC Virus Plaque Size in L Cells

Protamine sulfate concentration in regular agar overlay (μ g/ml)	Average plaque diameter after 72 hours incubation (mm)
0	0.2
50	0.8
75	2.7
100	4.2
200	6.0
500	6.5
1000	6.4

is inhibited by agar. Some other factor, then, must be responsible for the fact that, under methylcellulose overlay, EMC virus produces larger plaques than do any of the Mengo variants. In the absence of any data on burst size, affinity for L cells, etc., for the EMC virus, the reason for this can only be speculated upon, but one possibility is considered in the Discussion.

Plaque sizes under agar and agar components

Agarose. Once it had been established that agar inhibited plaque development by M- and S-Mengo, it became of interest to study the effect of individual agar components on plaque size. Agar is a complex substance, consisting of at least two polysaccharides, agarose and agaropectin. Agarose appears to have a linear structure made up of alternating residues of β -D-galactopyranose and 3,6-anhydro- α -L-galactopyranose, and contains few, if any, charged groups (Araki, 1959). The structure of agaropectin is not well understood, and it may possibly be a mixture of several polysaccharides. As a generalization, it differs from agarose in that it is sulfated, and contains, in addition to the constituents of agarose, D-glucuronic acid and pyruvic acid (Araki, 1959).

Agarose, which constitutes about two-thirds of the total weight of agar, was prepared as described in Materials and Methods, and was used in place of agar in an overlay which otherwise was identical to regular agar overlay. Plaque sizes of the three Mengo variants under varying concentrations of agar and agarose were examined, and the results are summarized in Figure 5.2. It can be seen that both M- and S-Mengo produce

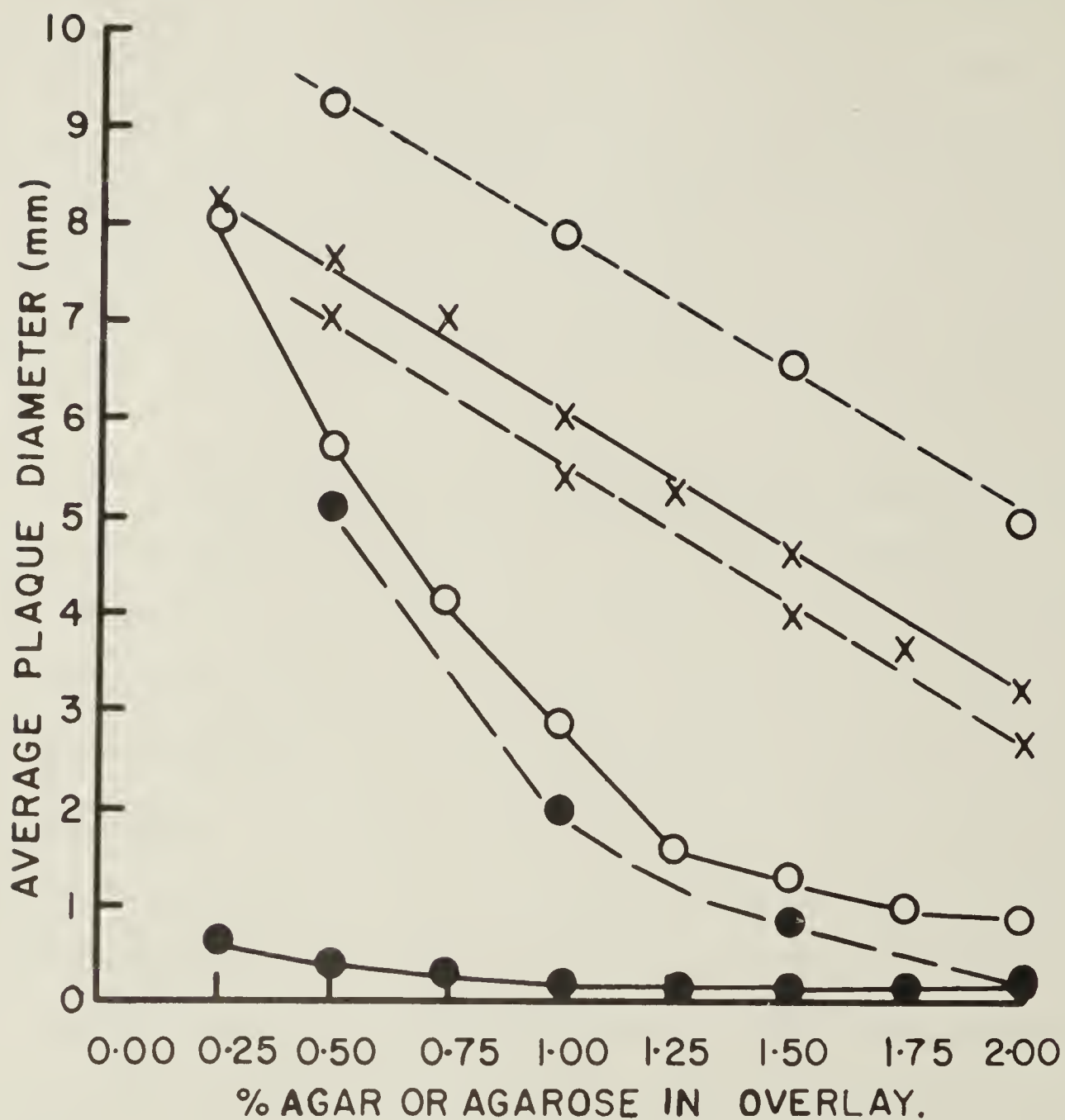


Figure 5.2. Plaque sizes of the Mengo variants under overlays containing varying concentrations of Noble agar or agarose. Solid lines: Noble agar. Broken lines: agarose. ● = S-Mengo; ○ = M-Mengo; x = L-Mengo.

much larger plaques under agarose than they do under the same concentration of agar. L-Mengo, on the other hand, produces slightly smaller plaques under agarose than under agar, an observation that may be explained by a slightly greater gelling capacity of the former. The linear relationship between gel concentration and L-Mengo plaque size under both overlays indicate that diffusion of the virus particles is not inhibited by the sulfated polysaccharide present. M-Mengo plaques developed under agarose show the same linear dependency on gel concentration, again indicating no inhibition of diffusion, but under agar the slope of the curve is markedly increased. S-Mengo still gives "inhibited" plaques under agarose. This, however, may simply be a reflection of the marked sensitivity of this variant to the agar inhibitor, which may still be present in trace amounts in the agarose preparation. That the addition of protamine to the agarose overlay results in a further increase of S-Mengo plaque size corroborates this suggestion.

One further point that may be worth noting is that the average size of plaques produced by M-Mengo under agarose is significantly larger than that produced under agar containing optimal quantities of protamine (7.9 mm and 6.3 mm respectively, after 72 hours incubation under 1% gels). It seems, therefore, that protamine cannot completely reverse the inhibition imposed by the agar factor.

Washed agar. Vogt et al. (1957) described a class of poliovirus variants which form fewer plaques under agar overlay with a low bicarbonate content than under overlays

containing higher concentrations of NaHCO_3 . Agol and Chumakova (1963) have presented data which show that the multiplication of these so-called d variants is inhibited by a sulfated polysaccharide, which can be removed from agar by saline extraction, and that the d marker cannot be demonstrated when such washed agar is used in the overlay. Data were presented in the previous chapter concerning the inhibitory effect of a sulfated polysaccharide (extracted from agar by a method similar to that employed by the Russian group) on the interaction of the Mengo variants and L cells. Since the preparation of agarose is rather laborious, the effect of washing agar, as a possible means of obtaining an inhibitor-free preparation, was examined.

Noble agar was shaken overnight with molar sodium chloride as described for the preparation of agar factor. This procedure was repeated five times, until the filtered supernatant gave no detectable precipitate upon addition of two volumes of ethanol. The agar was freed of chloride by repeated washing in distilled water, and was then dried with ethanol and ether. Plaque sizes of L-Mengo under an overlay containing this washed agar did not differ significantly from those obtained under regular agar overlay; those of M-Mengo were increased twofold in diameter, while only a slight increase was noted in those of S-Mengo. It appears, then, that although saline washing removes some inhibitor, agar contains material inhibiting both M- and S-Mengo plaque development which cannot be removed by this means.

Table 5.2

Plaque Sizes of Mengo Variants under Agar and Agar Components

Gelling medium (1.0%)	Average plaque diameter after 72 hours' incubation (mm)		
	S-Mengo	M-Mengo	L-Mengo
Noble agar	0.2	2.8	6.0
Bacto agar	0.2	1.5	5.9
Washed agar ^a	0.3	5.5	6.0
Agaropectin ^a	0.2	3.6	6.2
Agarose ^a	2.0	7.9	5.5
Agarose ^b	0.8	5.4	6.0
Noble agar containing 500 µg protamine/ml	6.0	6.3	6.0

^aPrepared from Noble agar as described in the text.

^bA commercial preparation; Industrie Biologique Française S.A., Gennevilliers (Seine), France.

Agaropectin. The effect of the sulfated polysaccharide, agaropectin, on plaque size was next examined. Acetylated agaropectin, formed during the preparation of agarose, was shaken with chloroform again to remove any acetylated agarose still present, and was deacetylated under the same conditions as was the latter (see Materials and Methods). It was then washed several times with molar sodium chloride, followed by washing with distilled water. The result was a pale brown powder which had a similar, though perhaps slightly less, gelling capacity to agar. Plaque sizes of the variants under this material did not differ greatly from those obtained under normal agar overlay.

The results of these investigations are summarized in Table 5.2. It is concluded that there are at least two components in agar capable of inhibiting the development of plaques by M- and S-Mengo, only one of which can be extracted with saline solution. The inhibition due to both fractions, however, can be relieved by the addition of protamine to the overlay medium.

Effect on plaque sizes of the addition of polyanions to regular agar overlay

As can be seen from the current literature, considerable effort is being made to explain the varied effects of polyanions, both synthetic and biological, on viral growth. At present, little is known about the actual mechanisms whereby they inhibit (or in some cases, enhance) infection, but they appear to block initial virus-cell interaction (Liebhaber and Takemoto, 1963; Schulze and Schlesinger, 1963; Schulze, 1964;

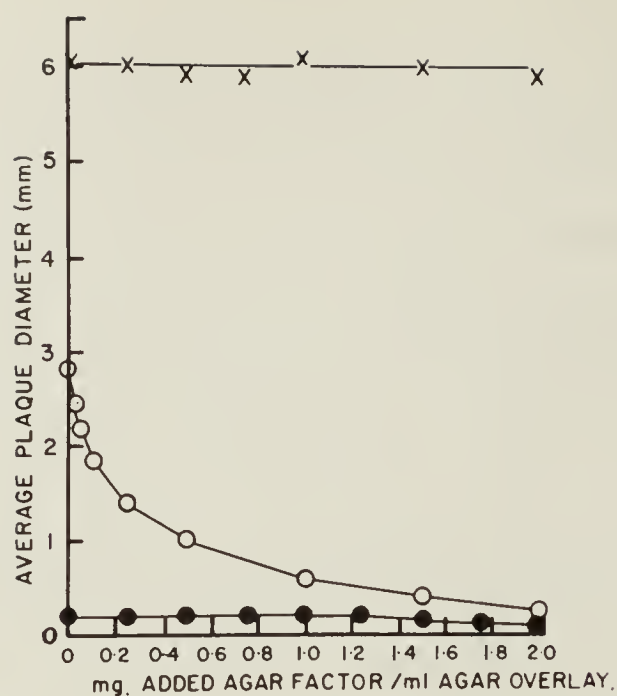


Figure 5.3. Plaque sizes of the Mengo variants under regular agar overlay containing varying concentrations of saline-extracted sulfated agar polysaccharide (agar factor). ● = S-Mengo; o = M-Mengo; x = L-Mengo.

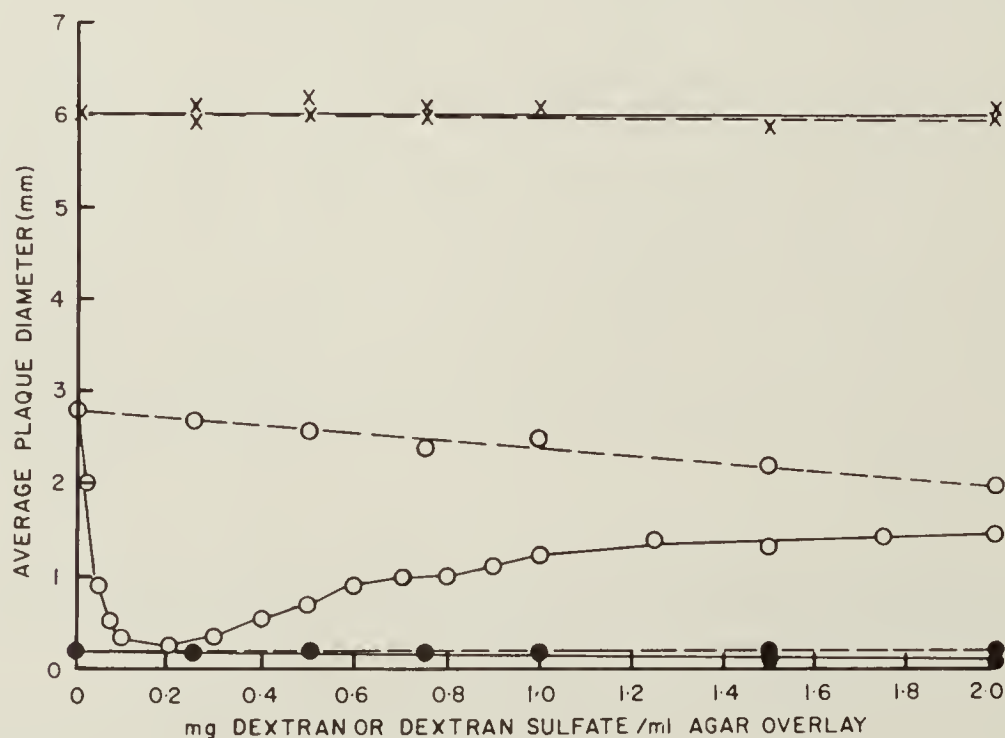


Figure 5.4. Plaque sizes of the Mengo variants under regular agar overlay containing varying concentrations of dextran or dextran sulfate. Solid lines: dextran sulfate (Pharmacia; mol. wt. 500,000). Broken lines: dextran (Abbott; mol. wt. 80,000). ● = S-Mengo; o = M-Mengo; x = L-Mengo.

Bengtsson, 1965; etc.). In this section, the effect of poly-anions on a single parameter, that of plaque size, has been studied, and the results are summarized in the following paragraphs. It should be noted that no effect on plaque numbers was found with any of the compounds tested.

Figure 5.3 illustrates the effect of adding saline-extracted sulfated agar polysaccharide (agar factor) to regular agar overlay on the sizes of plaques produced by the variants. The plaque size of M-Mengo is considerably inhibited, but that of L- and S-Mengo is little affected, even at concentrations of 2 mg added agar factor per ml of overlay. This probably explains why M-Mengo produces smaller plaques under Bacto agar than under Noble agar (see Table 5.2), since about twice as much sulfated polysaccharide can be extracted by saline washings from the former type of agar as from the latter.

The use of agar factor in studying virus-polysaccharide interaction has certain drawbacks, the main one being that its chemical structure is not well understood. The synthetic sulfated polysaccharide dextran sulfate is more fully characterized chemically, and would seem, therefore, to be a better model compound for such a study. It was reasoned that since both the agar factor and dextran sulfate are anionic sulfated polymers, they would exhibit similar inhibitory properties towards Mengo virus. That this was not exactly the case can be seen from Figure 5.4 which illustrates the effect of addition of dextran sulfate 500 (Pharmacia) to the regular agar overlay on the sizes of plaques produced by the three variants. It is clear that this polyanion has no effect whatsoever on L-Mengo

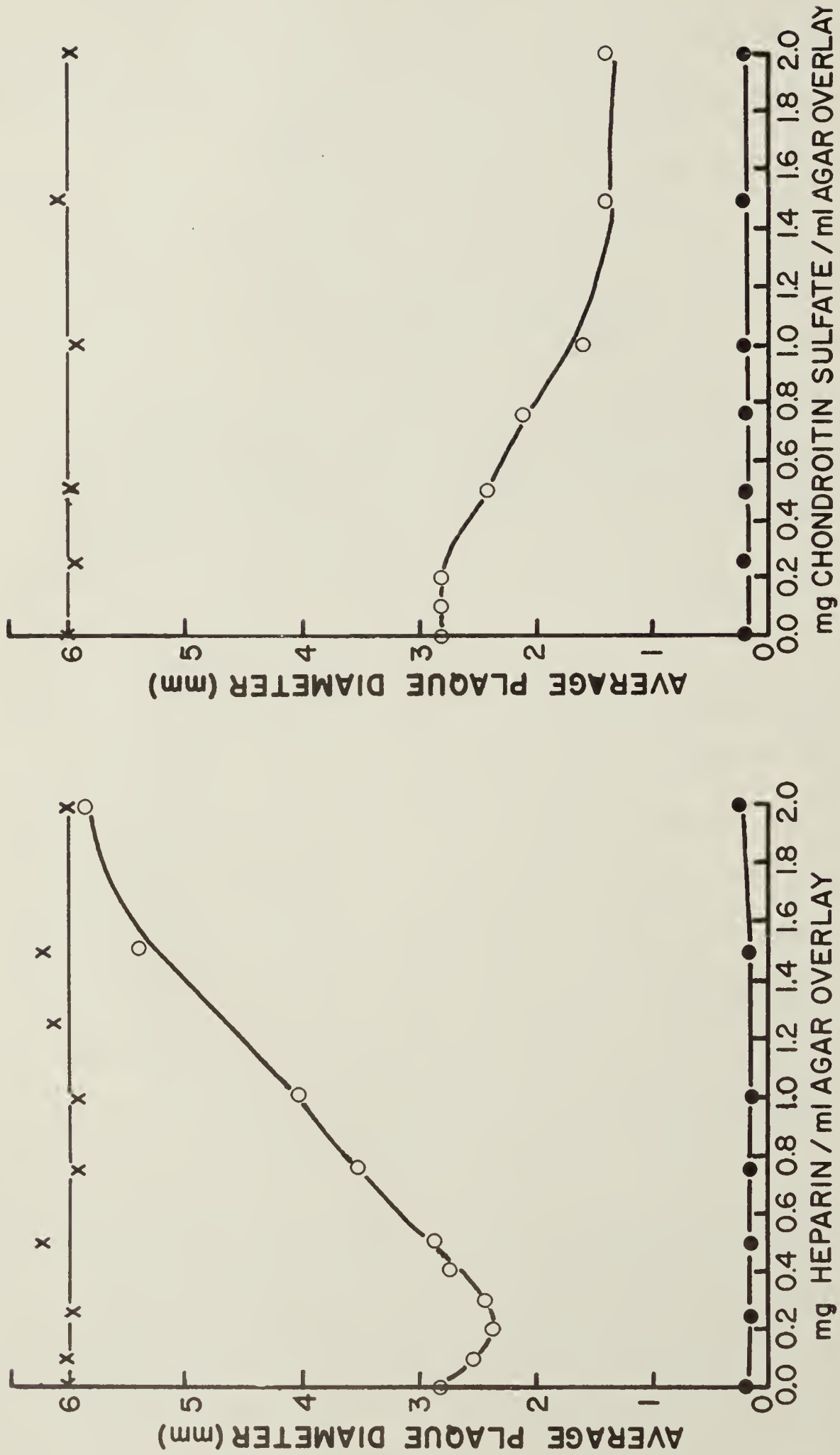


Figure 5.5. Plaque sizes of the Mengo variants under regular agar overlay containing differing concentrations of heparin or chondroitin sulfate. ● = S-Mengo; ○ = M-Mengo; x = L-Mengo.

plaque size over the range of concentration studied, and that S-Mengo plaque development is inhibited to even a greater extent than it is under unsupplemented agar overlay. These results with L- and S-Mengo are to be expected on the basis of what has been demonstrated with the added agar factor, but the effect on M-Mengo is somewhat surprising. Concentrations of dextran sulfate from 0-200 $\mu\text{g/ml}$ increasingly inhibit plaque size, but above this concentration, plaque development is less strongly inhibited. That this effect appears to be due to the presence of sulfated groups in the polyanion is shown by replacing dextran sulfate by dextran (Abbott) itself. In the presence of this material, M-Mengo plaque size is inhibited, but the degree of inhibition is not nearly as marked as that observed with the sulfated form (Figure 5.4).

The unexpected results with M-Mengo prompted an examination of the properties of other polyanions. Results obtained with heparin are shown in Figure 5.5. Again no change in L-Mengo plaque size was observed over the range of concentration studied, and little effect was seen in the case of S-Mengo. The slight inhibition of M-Mengo plaque size at concentrations between 0-200 μg heparin/ml was quite reproducible, but above these levels, from 300-2000 $\mu\text{g/ml}$, heparin can be seen to increase plaque size to over twice the diameter obtained under regular agar overlay alone. Chondroitin sulfate, on the other hand, has no enhancing effect, and even inhibits M-Mengo plaque size slightly (Figure 5.5). As with all other polyanions tested, little or no effect was noted on L- and S-Mengo plaque sizes.

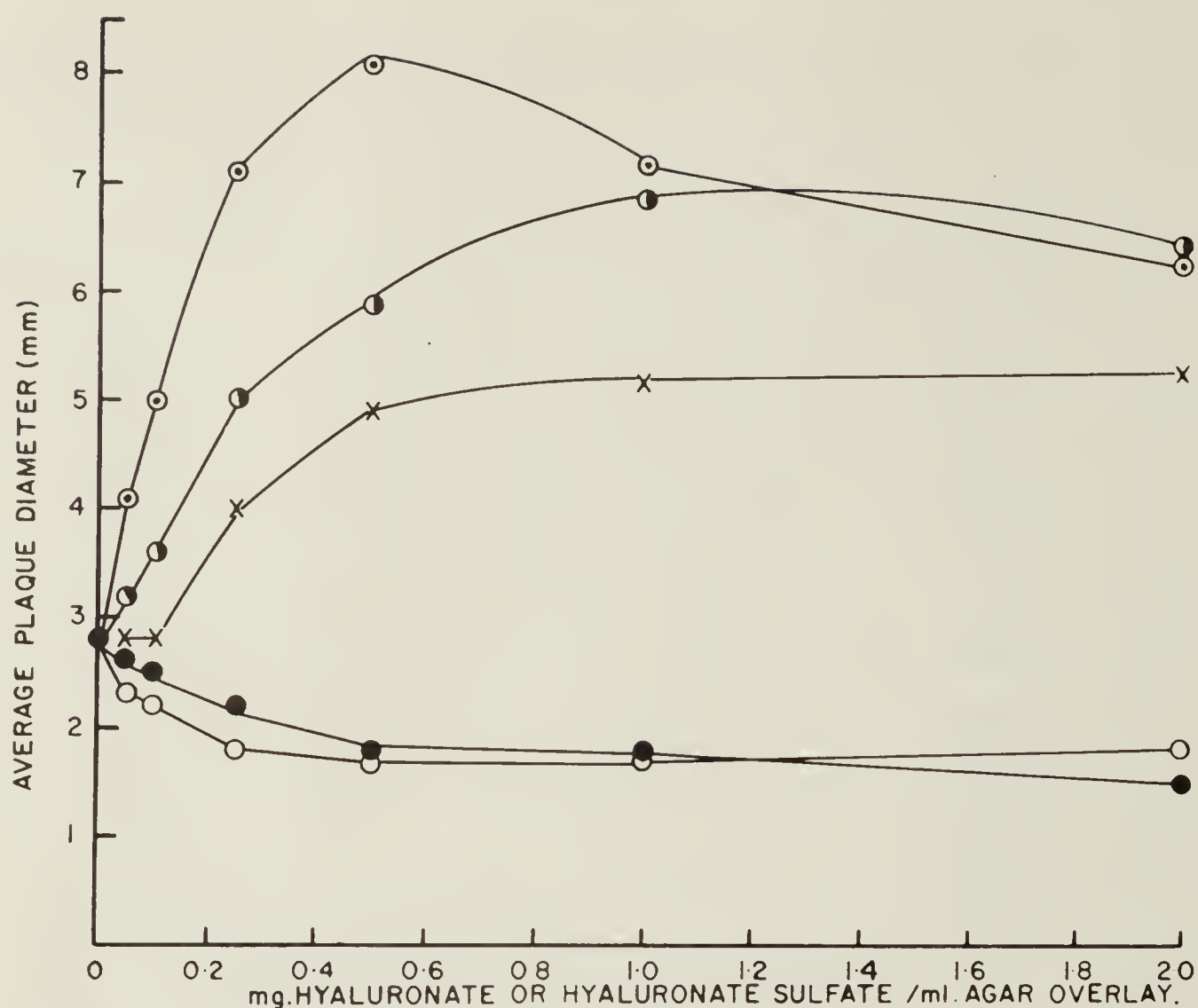


Figure 5.6. Plaque sizes of M-Mengo under regular agar overlay containing varying concentrations of hyaluronate or its sulfated derivatives. ● = unsulfated hyaluronate; ○ = SN-137; x = SN-139; ● = SN-141; ○ = SN-143.

Table 5.3

Chemical Properties of Sulfated Hyaluronate Fractions

Fraction no.	SN-	Intrinsic viscosity	Hexuronic acid reducing groups (moles/mole)	Sulfate content % sulfur	moles*
137		0.330	94	13.7	2.0
139		0.105	80	13.3	1.9
141		0.057	52	13.1	1.9
143		0.048	26	13.2	1.9

* per mole of hexose.

Samples of hyaluronic acid and of sulfated derivatives (SN-137, -139, -141 and -143) thereof were obtained from Dr. D. L. Cook of G. D. Searle & Co., Chicago. The preparation, chemical and biological properties of these fractions have already been described by Cook et al. (1963). The sulfated derivatives all contain approximately the same amount of sulfate (3.80-3.97 moles per mole of hexosamine) but differ in molecular weight (SN-137 > 139 > 141 > 143; see Table 5.3, adapted from Table 1, Cook et al., 1963). Figure 5.6 summarizes the results of a study of their effects on M-Mengo plaque size. Quite clearly, enhancement of plaque size increased with decreasing molecular weight. The unsulfated material itself and the sulfated derivative of highest molecular weight had only mildly inhibitory effects, similar to that of dextran (refer to Figure 5.4).

Since molecular weight had such a profound effect on the ability of the sulfated hyaluronates to enhance M-Mengo plaque size, it was reasoned that dextran sulfates of molecular weights lower than that of the sample used earlier (see Figure 5.4) might also have plaque enhancing properties. This was tested with a series of dextran sulfates of molecular weights ranging from 15-20,000 to 5-40 million. Data provided by the supplier (Sigma) are listed in Table 5.4. Sulfopolyglucin (Riker), a glucose polymer with 1:4 α and 1:6 α linkages containing 2-3 sulfate groups per glucose unit (Windsor and Cronheim, 1961; Freeman, 1964) was used to extend the lower limit of this series. It contained 12-15 glucose residues, and therefore had a molecular weight of about 6,000.

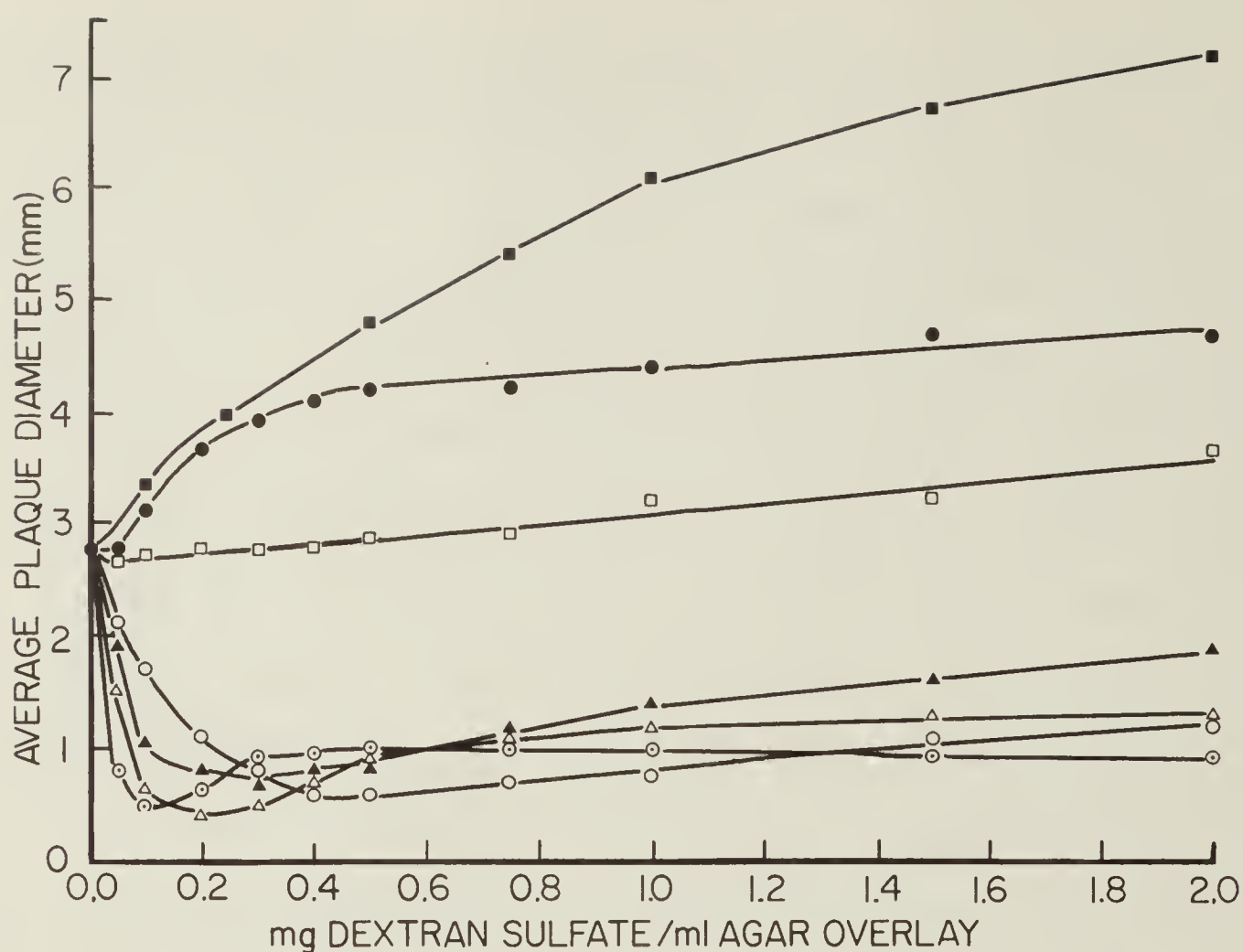


Figure 5.7. Plaque sizes of M-Mengo under regular agar overlay containing varying concentrations of dextran sulfates of differing molecular weights. ■ = sulfopolyglucin; ● = type 15S; ○ = type 60S; ▲ = type 200S; △ = type 500S; ⊙ = type 2000S; □ = type 5-40S.

Table 5.4

Chemical Properties of Dextran Sulfate Fractions

Type	Molecular weight	Sulfate content	
		% sulfur	moles*
15S	15-20,000	14.3	1.6
60S	60-90,000	13.5	1.5
200S	200-300,000	12.7	1.4
500S	500,000	17.0	1.9
2000S	2,000,000	17.0	1.9
5-40S	5-40,000,000	11.9	1.3

* per mole of hexose.

These fractions had advantages over the sulfated hyaluronates in that they were of accurately known molecular weights and were available in much larger quantities. Results arising from these studies are shown in Figure 5.7. Of the seven preparations tested, only the sulfopolyglucin and the dextran sulfate of lowest molecular weight (15-20,000) enhanced M-Mengo plaque size. Over the range 0-100 $\mu\text{g/ml}$, the others inhibited plaque size increasingly with increasing molecular weight, and all gave lesser degrees of inhibition at concentrations above 200 $\mu\text{g/ml}$. The only exception to this generalization was the fraction of molecular weight 5-40 million, which had little effect on plaque size over the whole concentration range studied, and even appeared to enhance it slightly.

On the basis of these results, it could be argued that the use of a single dextran of molecular weight 80,000 (Figure 5.4) has not adequately shown that sulfate groups are responsible for the observed effects. This point was reexamined using three dextrans (Sigma) of molecular weights 15-20,000, 150-200,000, and 5-40 million, corresponding approximately to three of the dextran sulfate fractions studied. It can be seen (Figure 5.8) that although inhibition of plaque development increased with increasing molecular weight of the dextran, the degree of inhibition was in no case marked, and no enhancement of plaque size was obtained. It is apparent, then, that low molecular weight polymers will not enhance M-Mengo plaque size unless they also contain charged groups.

The possibility that phosphate groups might have the same effect as sulfate groups was considered. Attempts to prepare

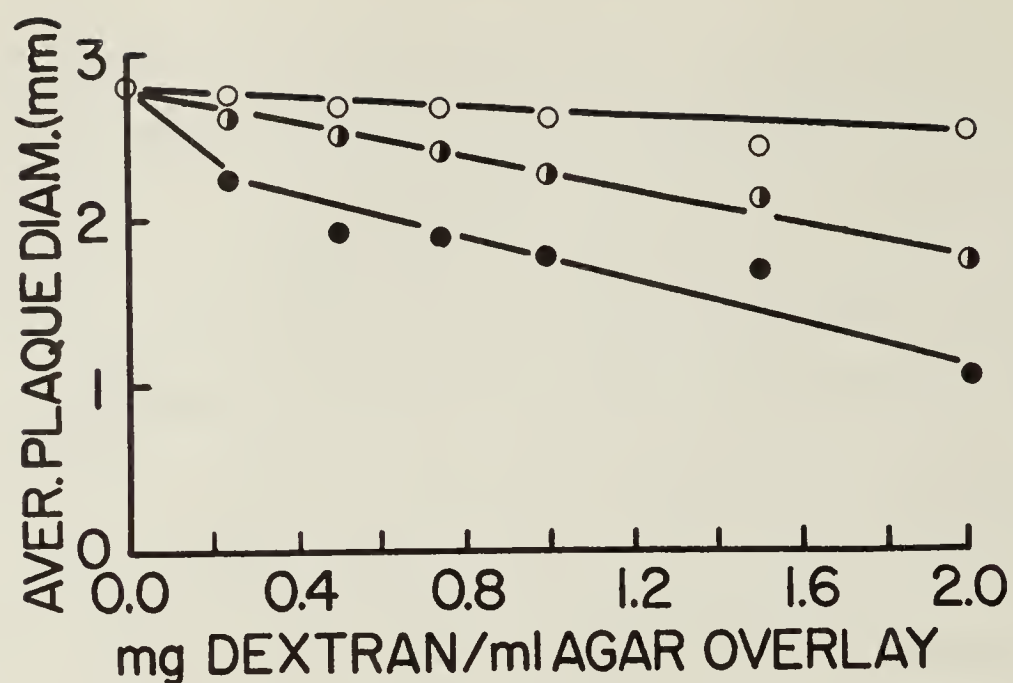


Figure 5.8. Plaque sizes of M-Mengo under regular agar overlay containing varying concentrations of dextran (Sigma). o = type 15; ◐ = type 150; ● = type 5-40.

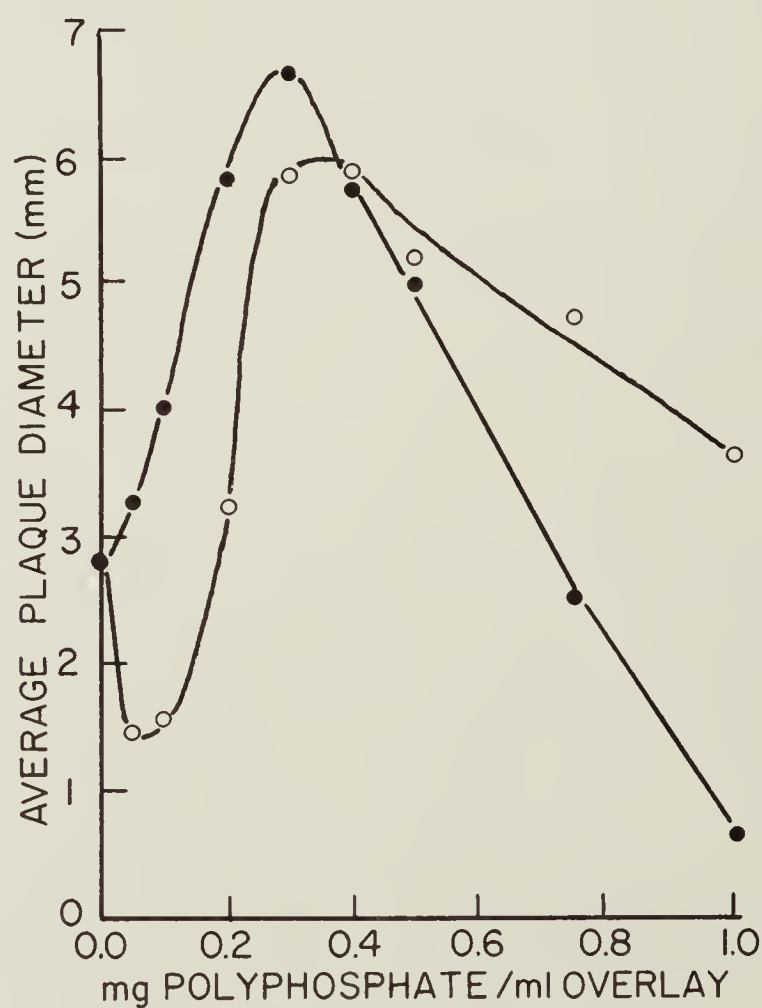


Figure 5.9. Plaque sizes of M-Mengo under regular agar overlay containing varying concentrations of polyphloreitin phosphate (●) and polyphloroglucinol phosphate (o).

dextran phosphates by treating dextrans with phosphorus oxychloride (Peterson and Sober, 1961) were not successful: the resulting preparations had very low phosphate contents (less than 0.1 phosphate group per hexose moiety) and had much the same effect on the size of plaques of M-Mengo as did the corresponding dextran. Samples of the synthetic polymers polyphloroglucinol phosphate and polyphloreitin phosphate, however, were obtained from Aktiebolaget Leo, Hälsingborg, Sweden. The polyphloroglucinol phosphate (Leo 137B) was polydisperse and contained mostly secondary phosphates; the polyphloreitin phosphate (Leo 101A) contained around 70% of its phosphorus content as primary phosphate, and had a molecular weight of 15,000. Both were developed originally as enzyme inhibitors (Diczfalusy et al., 1953; Fernö et al., 1953), and both have been shown to have an inhibitory effect on the attachment of influenza virus to chicken red blood cells by apparent combination with the virus (Penttinen, 1956, 1957). Polyphloroglucinol phosphate is also a potent inhibitor of herpes simplex virus replication (Vaheri and Penttinen, 1962). Figure 5.9 shows that both polyphosphates enhanced the plaque size of M-Mengo over a limited range of concentration. At low concentrations (less than 100 µg/ml) polyphloroglucinol phosphate quite reproducibly and quite strongly inhibited plaque size. It was not possible to investigate the effect on plaque size of concentrations of these polymers higher than 1 mg/ml, due to their high toxicity to L cells, but it seems clear that only further inhibition of plaque size and number would result.

One other series of polymers was tested: namely, dextrans of molecular weight 60,000 containing 0.5, 1 and 2 acid esters of phthalic acid per unit of glucose (Leo 128A, Leo 129A, and Leo 127A, respectively). Increased substitution of the aromatic carboxyl (phthalate) groups gave increased inhibition of plaque size, but even with Leo 127A the extent of inhibition was considerably less marked than with dextran sulfate of comparable molecular weight. The degree of inhibition was also proportional to the amount of inhibitor in the overlay.

Mechanism of enhancement of plaque size by polyanions

Evidence was presented in the previous chapter to indicate that the virus-inhibiting material of agar blocks cell-virus interaction by directly immobilizing the virus particles. It seems likely that the other polyanions tested here inhibit plaque size by a similar mechanism. However, this does not explain why the lower molecular weight polymers can enhance M-Mengo plaque size.

In order to explain the inhibition and enhancement of plaque size by a single polyanion, the hypothesis was advanced that two mechanisms may be involved (Campbell and Colter, 1965). It was postulated that the lower molecular weight polymers could penetrate the cell wall efficiently and that they had some intracellular mechanism resulting in a faster spread of virus in the cell monolayer. Higher molecular weight polymers were considered to be unable to penetrate the cell wall, or at least not efficiently, which limited their action to extracellular inhibition of virus-cell interaction. One possibility suggested for the hypothetical intracellular action of heparin

and the other lower molecular weight polymers was that they might function as ribonuclease inhibitors, thus removing one of the means whereby virus multiplication in the cell could be diminished (the ability of heparin to inhibit ribonuclease and other enzymes has been well documented (Engelberg, 1963)).

The hypothesis was open to direct experimental verification, and this was done using heparin and dextran sulfate (Pharmacia) as representative polyanions. These were used at concentrations of 2 mg/ml and 200-250 μ g/ml respectively, which were the concentrations giving maximal plaque enhancement (heparin) and maximal plaque inhibition (dextran sulfate) (see Figures 5.4 and 5.5).

Assuming the occurrence of the postulated intracellular action, a faster spread of virus under overlay containing heparin could be accomplished by either, or both, of two mechanisms: an earlier release of intracellular virus, or an increased production of infectious virus. Extracellular inhibition by dextran sulfate would result only in decreased production of virus, since initial virus-cell interaction would be considerably inhibited.

Single-cycle growth studies of M-Mengo in growth medium or in growth medium containing heparin or dextran sulfate failed to support this theory. In one experiment, the results of which are shown in Figure 5.10, a series of L cell monolayers was incubated at 37° for 30 minutes with a tenfold multiplicity of M-Mengo in 4 ml growth medium, or growth medium containing 2 mg/ml heparin or 200 μ g/ml dextran sulfate.

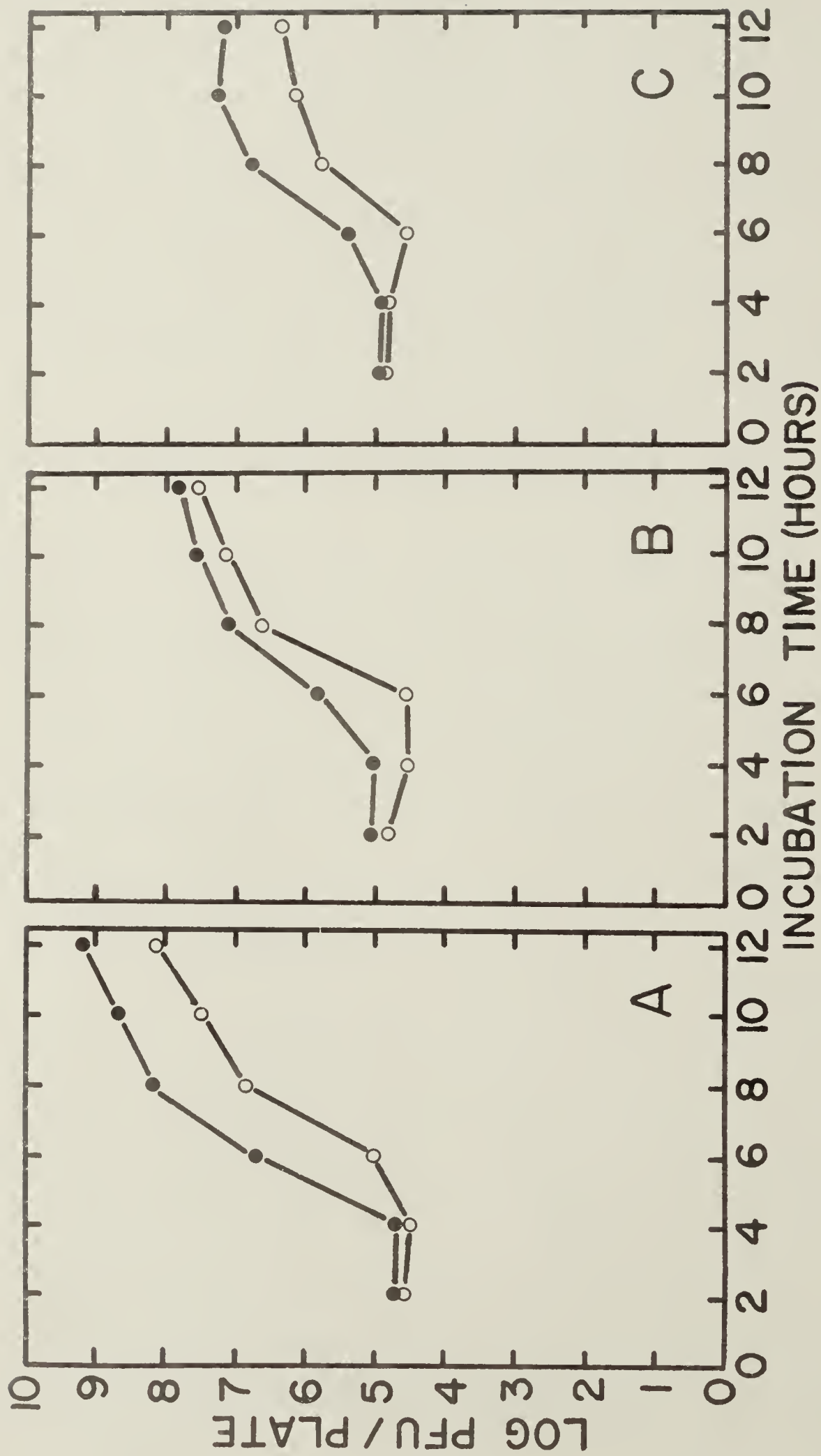


Figure 5.10. Single cycle growth curves of M-Mengo in L cell monolayers infected at high multiplicity. Closed symbols: total virus. Open symbols: extracellular virus. A. in growth medium alone. B. in growth medium + 200 µg dextran sulfate 500/ml. C. in growth medium + 2 mg heparin/ml.

Fluid was then removed from the plates, and the monolayers were washed and re-fed with fresh 4 ml aliquots of the same media (without virus). Incubation was continued at 37⁰, and duplicate monolayers of each group were removed at 2 hour intervals for a total of 12 hours. One monolayer of each pair was frozen immediately at -20⁰. It was thawed and re-frozen once more before its virus content was assayed to give a measure of the total virus produced. The fluid medium was removed from the twin monolayer by decanting, and, after chilling, was centrifuged at low speed to remove particulate material. This procedure allowed extracellular virus to be measured. Data acquired in this way (Figure 5.10) showed that intracellular virus was not released earlier from cells maintained in medium containing heparin than from cells incubated in growth medium alone. Moreover, less virus was produced in the presence of heparin than in the control, demonstrating that, in this system at least, heparin had an inhibitory effect only, similar to, but less marked than that of dextran sulfate.

This experiment seemed to rule out the possibility that intracellular virus may be released earlier into heparin-containing medium. Virus plaques, however, are produced by multiple growth cycles in cells under semisolid overlay--conditions quite different from those used here to examine a single cycle of multiplication in liquid media. Another experiment was designed, therefore, to follow virus production under semisolid overlays under conditions normally used for plaque production.

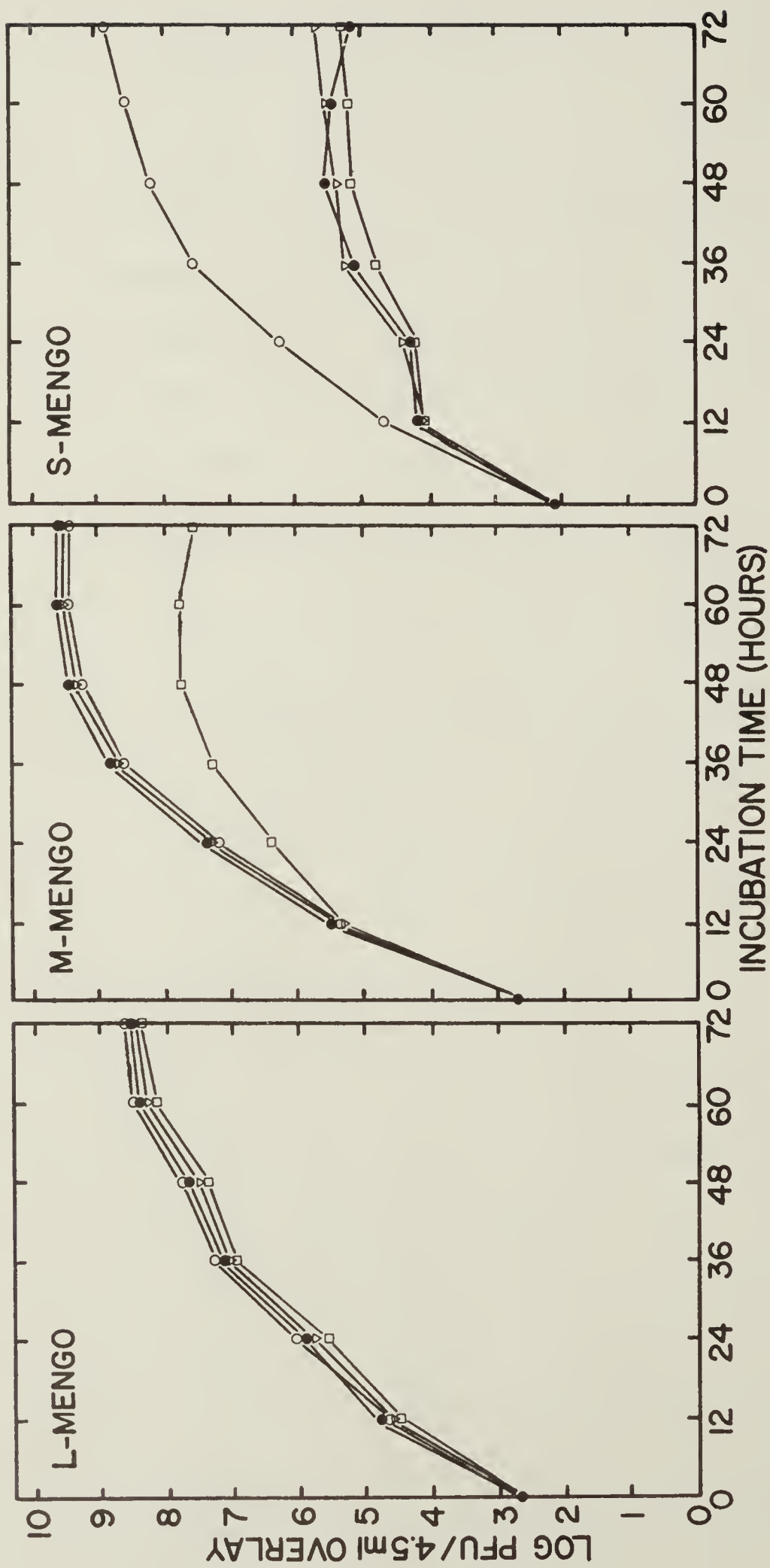


Figure 5.11. Multiple cycle growth curves of the Mengo variants in L cell monolayers under regular agar overlay, with or without the addition of polyions. ● = regular agar overlay; ○ = overlay containing 2 mg/ml of heparin; □ = overlay containing 250 µg/ml of protamine sulfate; ▽ = overlay containing 250 µg/ml of dextran sulfate (Pharmacia).

A series of L cell monolayers was infected with several hundred PFU of S-, M-, or L-Mengo. After an incubation period of 30 minutes, fluid was removed from the plates by suction and 4.5 ml of regular agar overlay, without additions or containing 2 mg/ml of heparin, 250 μ g/ml of dextran sulfate (Pharmacia), or 500 μ g/ml of protamine sulfate, was applied. Incubation at 37⁰ was continued. Monolayers were removed at 12 hour intervals for 72 hours and frozen at -20⁰. On thawing, the agar gels collapsed into liquid, and after one more cycle of freezing and thawing, this fluid was assayed for virus. Figure 5.11 shows the results of one such experiment. It is evident that the size of a virus plaque is not necessarily directly related to the amount of virus it contains. L-Mengo gives plaques of essentially the same diameter under all 4 overlays tested, and little difference was observed in the total amount of virus produced under each. The plaque size of M-Mengo is greatly reduced under overlay containing 250 μ g/ml of dextran sulfate (Figure 5.4) and this was reflected in a decrease in the total amount of virus present. However, under the regular agar overlay, or under overlay containing 500 μ g/ml of protamine, or 2 mg/ml of heparin, which produce M-Mengo plaques of 2.8, 6.3, and 6.0 mm diameter respectively, little difference was noted in the total virus produced. S-Mengo forms plaques of minute size under agar overlay or overlay containing either heparin or dextran sulfate, and comparable amounts of virus were produced under all three overlays. Addition of protamine to the overlay greatly increased both the plaque size and the amount of virus produced.

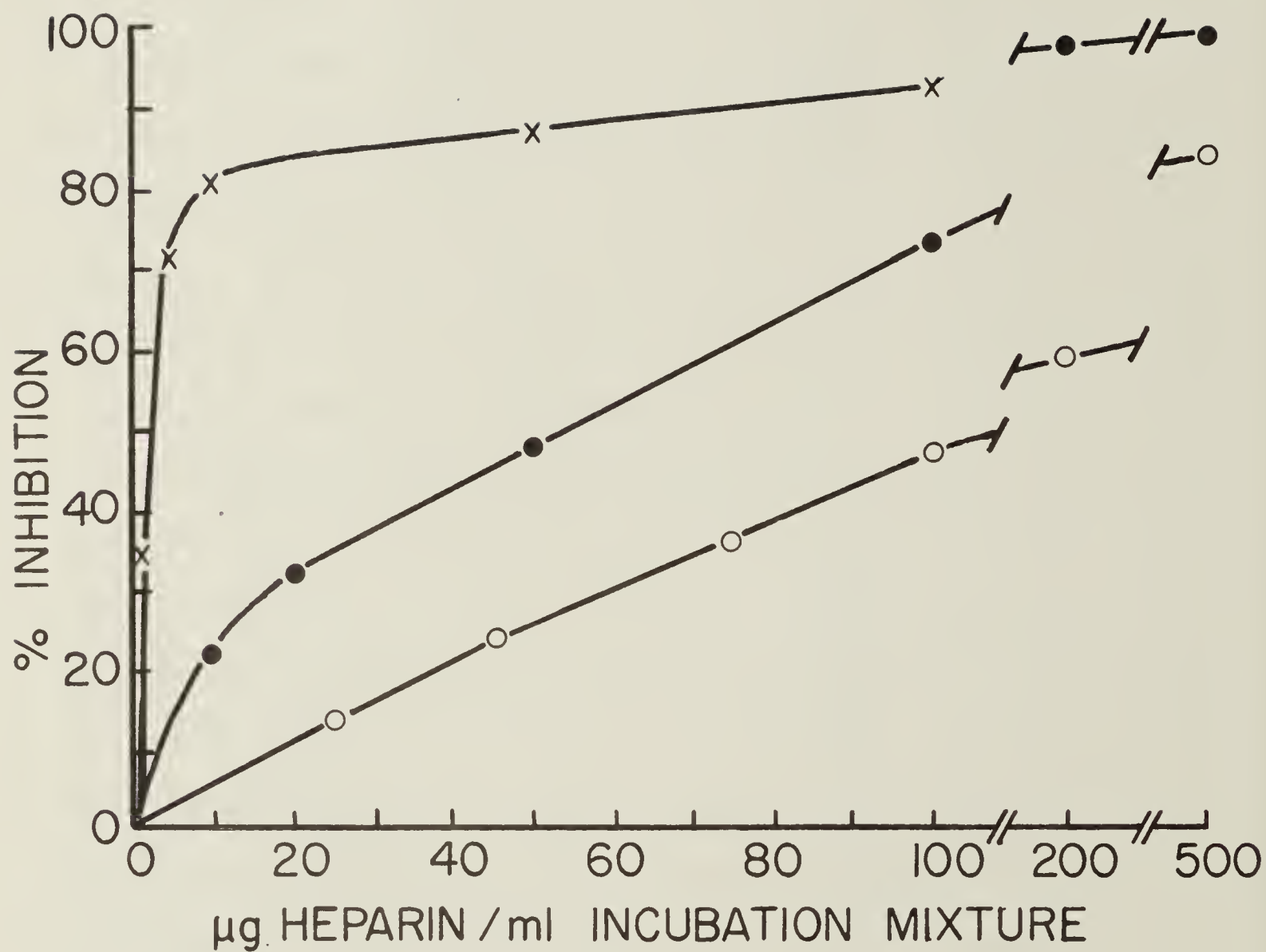


Figure 5.12. Inhibition of L cell-Mengo virus interaction in suspension by heparin. Incubation mixtures contained 200,000 cells/ml and an input virus multiplicity of 10. ● = S-Mengo; ○ = M-Mengo; x = L-Mengo.

Data provided by this experiment, then, appear to rule out completely the hypothesis that heparin enhances M-Mengo plaque size by increasing virus production. Other possible explanations were therefore sought.

Using the suspended cell system, the relative sensitivities of the variants to inhibition by heparin were determined. The experimental design employed in these studies was almost identical to that used to determine the relative sensitivities of the variants to agar factor and to protamine (Chapter 4). Briefly, L cells were suspended at a concentration of 200,000/ml in virus diluent pH 7.6 containing an input virus multiplicity of 10 and varying concentrations of heparin. Incubations were carried out at 25° for 30 minutes, after which the cells were spun down and washed with virus diluent before being assayed for infectious centers. The results of these experiments are summarized in Figure 5.12. Surprisingly, L-Mengo is considerably more sensitive to inhibition by heparin than are the other two variants. Expressed in terms of the concentration of heparin that reduces cell-virus interaction by 50%, the susceptibilities of S-, M-, and L-Mengo are approximately 50 µg, 110 µg, and 2 µg, respectively.

Table 5.5 compares the susceptibilities of the Mengo variants to heparin with their susceptibilities to agar factor, and protamine sulfate (data taken from Table 4.1). The widely differing sensitivities of the variants to heparin and to agar factor imply that the two polyanions bind to different sites on the virus particles. In the case of M-Mengo, heparin would seem to bind in such a way as to impede the formation of

Table 5.5

Inhibition of Infectious Center Formation
in Mengo Virus--L Cell Mixtures by
Heparin, Agar Factor, and Protamine.

Variant	Concentration of inhibitor producing 50% inhibition		
	Heparin ($\mu\text{g/ml}$)	Agar factor ($\mu\text{g/ml}$)	Protamine ($\mu\text{g/ml}$)
S	50	0.4	0.9
M	110	3.3	14.0
L	2	35	7.5

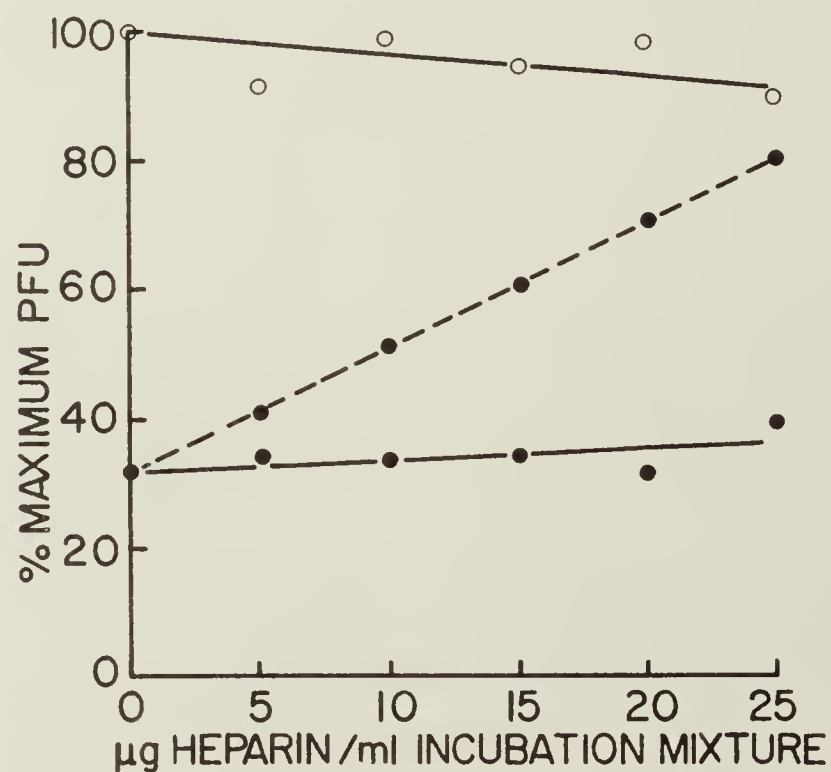


Figure 5.13. Effect of heparin on the inhibition imposed by agar factor on L cell--M-Mengo interaction in suspension. Incubation mixtures contained 200,000 cells/ml and an input virus multiplicity of 10. The suspending media consisted of virus diluent alone (o) or virus diluent containing 5 $\mu\text{g/ml}$ of agar factor (●). Broken line: data predicted by the proposed theory. Solid lines: experimental results.

infectious centers much less efficiently than does the agar factor. If the hypothetical sites at which the polyanions bind are close to one another, it is reasonable to speculate that a complex of virus and one of the polymers may not be able to bind the second, due to a partial or complete blocking of the site at which the second polymer would normally be bound. Under agar overlay containing heparin, this competition could occur, resulting in a faster spread of virus in the monolayer and more rapid development of plaques. Heparin and protamine would therefore both have plaque-enhancing ability by incapacitating the agar factor--the former by a form of competitive inhibition, and the latter by direct combination with the agar constituent.

This hypothesis was tested with M-Mengo in the suspended cell system under exactly the same conditions as were used for the determination of the relative sensitivities of the variants to inhibition by heparin. A series of cell-virus suspensions (200,000 cells/ml; input virus multiplicity = 10) were incubated for 30 minutes at 25° in the presence of various concentrations of heparin ranging from 0-25 µg/ml, and the number of infectious centers formed in each mixture was determined in the usual way. A second series, identical to the first except that each suspension contained 5 µg of agar factor/ml, was handled at the same time and in precisely the same manner. M-Mengo--L cell interaction is inhibited to the extent of 60-70% by an agar factor concentration of 5 µg/ml, whereas heparin at a concentration of 5 µg/ml produces negligible inhibition, and reduces the formation of infectious

centers by only 10-15% at a concentration of 25 $\mu\text{g/ml}$ (see Figure 5.12). Taking 50,000 and 10,000 as the molecular weights of the agar factor and heparin, respectively, a system containing 5 $\mu\text{g/ml}$ of agar factor and 25 $\mu\text{g/ml}$ of heparin contains 25 times as many molecules of heparin as agar factor. In the suspended cell system, then, any competition between heparin and agar factor for the virus particles under these conditions might be expected to favor the formation of a virus-heparin rather than a virus-agar factor complex. If this happened, one might reasonably predict that the inhibition of formation of infectious centers imposed by the agar factor would be at least partially overcome by the presence of heparin. Figure 5.13 illustrates what the outcome of this experiment was expected to be, based on the above reasoning, and what was actually found. The results indicate that heparin is completely unable to relieve the inhibition imposed by the agar factor. The hypothesis that heparin enhances the plaque size of M-Mengo by competing with agar factor for the virus particles appears, therefore, to be untenable. It is tempting to conclude that in the presence of heparin, plaques of M-Mengo, like Topsy, "just grewed".

Discussion

The use of methylcellulose as a substitute for agar has the advantage that there are no sulfated polysaccharides present to inhibit certain types of virus. This has resulted in the preferential use of the former as a gelling medium in the plaque assay of some types of viruses. For example, Rapp

(1963) found that under methylcellulose overlay, plaques formed by herpes simplex virus could be counted sooner, plaques became larger, and more plaques appeared than under agar. Schulze and Schlesinger (1963a) used a methylcellulose overlay to obtain plaques with type 2 dengue and other group B arboviruses which did not form plaques in agar-overlaid monolayers. Bengelsdorff (1963) used methylcellulose overlay in the titration of foot and mouth disease virus, and Hövel and Günther (1964) employed a methylcellulose medium in the formation of plaques of poliovirus. Nevertheless, this medium has its own drawbacks, not the least being that it is inconvenient to handle. Results presented here also show that it is not a good medium for use with the Mengo virus--L cell system, due to the small sizes of plaques obtained with it. From a practical point of view, there seems to be little gained from using methylcellulose, since agarose appears to offer all its advantages without having any of its disadvantages.

Starch gel has recently been introduced for use in the plaque assay of animal viruses. Though more cumbersome to prepare than agar, and though not as flexible as agarose, it has the advantage over methylcellulose of becoming just as solid as agar (DeMaeyer and Schonke, 1964).

There are, then--in addition to methylcellulose--at least two types of gelling medium available which are free of sulfated material. Moreover, since the inhibitory action of sulfated agar polysaccharides can be almost completely blocked by protamine or DEAE dextran, agar to which optimal amounts of

one of these polycations has been added is, in effect, an inhibitor-free medium. The effectiveness of the latter and the ease with which it may be prepared probably makes it the most useful overlay for the assay of virus strains which are sensitive to polyanions. Agar containing 250 μ g protamine/ml is used in this laboratory for all plaque titrations of S-Mengo.

In view of the fact that about twice as much sulfated polysaccharide can be extracted by saline washings from Bacto agar as from Noble agar, the latter would appear to be more suitable for use in virus plaque assays. Nevertheless, it has been reported that reo-1 and certain ECHO viruses fail to grow under Noble agar, but readily produce plaques under Bacto agar (Hsiung and Melnick, 1957; Melnick, 1962). In addition, Nagai and Hammon (1964) reported that plaque numbers of Japanese B encephalitis virus grown in hamster kidney monolayers were slightly higher under Bacto agar than under Noble agar overlay, and that the cells survived longer under the former. These observations appear to be completely at variance with the present ones, and would imply that sulfated agar polysaccharides may enhance plaque formation by these viruses. This is not completely improbable, however, since the size of plaques produced by some strains of poliovirus is in fact increased by the addition of dextran sulfate to the overlay (Takemoto and Liebhaver, 1962). This point will be discussed later.

The reason that EMC virus produces larger plaques under methylcellulose overlay than do any of the Mengo variants is

not clear, especially since EMC virus is closely related to Mengo. One would have expected the two types of virus to behave similarly under both overlays. This is not the case, however, and some observations of Dales and Franklin (1962) may help to provide an explanation. These workers have shown that changes in the permeability of L cells infected with EMC virus commence much earlier than in L cells infected with Mengo virus. Virus in EMC-infected monolayers may be allowed therefore, to spread more rapidly than virus in Mengo-infected ones. This cannot be the whole story, however, since it does not explain why the relative plaque sizes of the Mengo variants are essentially the same under methylcellulose as they are under an agar overlay. This point has also been noted in another virus system. Ushijima et al. (1962) have found that the relative plaque sizes of plaque mutants of WEE virus were not affected by substitution of methylcellulose for agar. Inhibition due to agar could, however, be reversed by the addition of a polycation--in this case, DEAE Sephadex--to the agar overlay.

Data have been presented to show that agar contains at least two fractions capable of inhibiting plaque development by M- and S-Mengo. Repeated saline washing of Noble agar removes a total of 3-4% of the weight of agar as a homogeneous material--the agar factor. The gelling capacity of this substance is poor, which distinguishes it from the rest of the sulfated fraction of whole agar, the agaropectin. A 1% aqueous solution of agaropectin, as prepared by the method described earlier, forms a firm gel. Russell et al. (1964)

have used 1% agarose gels prepared by another method, for use in electrophoresis. The value of 1.10% reported by Araki (1959) as the minimum concentration of agarose capable of forming a gel, appears therefore to be rather high. The preliminary chemical analysis of the agar factor presented in Chapter 4 indicates that it is of similar composition to agarose, and it is possible that the difference between the two fractions is simply one of molecular weight.

Sands and Bennett (1964) have also reported the presence of two inhibitory fractions in agar; one water-soluble, the other water-insoluble. These were prepared by shaking agar gel with water, and probably correspond to what are termed herein, agar factor and washed agar. These fractions, of which the water-soluble one was the more potent, had a detrimental effect on the ability of a variety of phenols to inhibit the growth of Staphylococcus aureus. It is possible that the inhibitory activity of agar towards a number of antimicrobial agents (see Sands et al., 1963 for references) is due in part to the presence in agar of sulfated polysaccharides, and that these bind phenols, fatty amines (Hanus and Bennett, 1964) and other compounds in a manner analogous to that by which they bind Mengo virus.

The observation that the addition of some polyanions to agar overlay can increase virus plaque size is not entirely new, since Takemoto and Liebhaver (1962b) and Voss (1964) have reported enhancement of plaque formation of several poliovirus strains by dextran sulfate. Takemoto and Liebhaver (1962b) have also provided evidence for an intracellular site of action

Table 5.6

Effects of Sulfated Polysaccharides on M-Mengo Plaque Size Under Agar Overlay

Polymer	Molecular Weight	Approx. Sulfate Content (groups per hexose moiety)	Effect on M-Mengo Plaque Size
Dextran	10,000 - 40 million	0	Slightly inhibits, 0-2000 $\mu\text{g/ml}$
Hyaluronate	100,000	0	Slightly inhibits, 0-2000 $\mu\text{g/ml}$
Agar factor	50,000	0.15	Inhibits, 0-2000 $\mu\text{g/ml}$
Chondroitin sulfate	43,000	0.5	Slightly inhibits, 0-2000 $\mu\text{g/ml}$
Heparin	10-12,000	1.25	Slightly inhibits, 0-200 $\mu\text{g/ml}$ Greatly enhances, 300-2000 $\mu\text{g/ml}$
Sulfopolyglucin	6,000	2-3	Greatly enhances, 0-2000 $\mu\text{g/ml}$
Dextran sulfate	15-20,000	1.6	Enhances, 0-2000 $\mu\text{g/ml}$
	60,000 - 2 million	1.4-1.9	Greatly inhibits, 0-200 $\mu\text{g/ml}$ Inhibits, 300-2000 $\mu\text{g/ml}$
	5-40 million	1.3	No effect, 0-2000 $\mu\text{g/ml}$

of this sulfated polymer. They have shown for a type 1 (Mahoney) poliovirus strain, that gave plaques of enhanced size under agar overlay containing dextran sulfate, virus was released from the cells earlier and in greater amounts in the presence than in the absence of this polyanion. It is clear from Figures 5.10 and 5.11, however, that the enhancement of M-Mengo plaque size by heparin and low molecular weight dextran sulfates cannot be explained on this basis.

Enhancement of poliovirus plaque formation was obtained by Takemoto and Liebhaver (1962b) using a dextran sulfate of molecular weight 500,000 or 2 million (the authors do not state which). With M-Mengo, dextran sulfates of a molecular weight of 60,000 or more gave only inhibition of plaque size (Figure 5.7). Molecular weight is obviously a critical factor in determining whether a polyanion will enhance or inhibit the plaque size of M-Mengo, and from the data in Figure 5.7 it appears that the "balance point" lies between molecular weights of 20,000 and 60,000, probably nearer the lower figure. This may explain why heparin (molecular weight 10-12,000; Freeman, 1964) enhances M-Mengo plaque size, whereas chondroitin sulfate (molecular weight 43,000; Mathews and Dorfman, 1953), a substance of similar chemical structure, inhibits slightly.

Table 5.6 summarizes the properties of the sulfated polymers tested. These data, together with that obtained with the polyphosphates and dextran phthalates, indicate that the backbone structures of the polyanions do not appear to contribute to their inhibitory or enhancing effect on M-Mengo plaque size. The important factors appear to be molecular weight,

and the presence of a strong electronegative charge. Carboxyl groups (as in hyaluronic acid or the dextran phthalates) can not replace the strongly electronegative sulfate groups, although phosphates may. Nahmias et al. (1964) investigated the inhibitory effect of a number of synthetic and biological sulfated polymers on herpes simplex virus. They found that the viral inhibitory effect depended, within limits, on the degree of sulfation and on the size of the molecule, and was independent of the polysaccharide portion of the molecule, with respect to either the degree of branching, or the type of glycosidic or sulfur bonds it contained. Vaheri et al. (1964) made similar observations concerning the inhibitory effects on herpes virus of a wide variety of polymers.

The mechanism whereby low molecular weight polyanions, both sulfated and phosphorylated, enhance M-Mengo plaque size is obscure. On the other hand, the mechanism by which higher molecular weight dextran sulfates inhibit plaque development appears to be reasonably straightforward, namely, electrostatic binding, resulting in a decreased spread of virus. In Figure 5.7, with the inexplicable exception of the type 5-40S material, the degree of inhibition of plaque size over the range 0-200 $\mu\text{g/ml}$ is directly proportional to the molecular weight of the dextran sulfate. Styk and Rada (1964) have also found with a series of dextran sulfates that their efficiency as myxovirus inhibitors increased with increasing molecular weight. Yet the lessened degrees of inhibition imposed by the dextran sulfates at concentrations of greater than 200 $\mu\text{g/ml}$ cannot be accounted for so readily. One possible explanation

is that the preparations tested contained small percentages of low molecular weight material which could partially reverse the inhibitory actions of the major fractions when present in the overlay in sufficient quantity. The slight inhibitory action of heparin over the range 0-200 $\mu\text{g/ml}$ could likewise be due to a small amount of higher molecular weight impurities.

Some support for this theory comes from the data obtained with the polyphosphates, polyphloroglucinol phosphate, and polyphlorethin phosphate (Figure 5.9). The former was polydisperse, and its effect on M-Mengo plaque size consisted of an initial sharp inhibition (0-50 $\mu\text{g/ml}$) followed by considerable enhancement. These effects could be due to the competing action of the high and low molecular weight components, with the effect of the higher molecular weight components predominating at low concentrations, and the plaque-enhancing properties of the low molecular weight material overcoming this inhibition at higher concentrations. Polyphlorethin phosphate, on the other hand, was monodisperse, with a molecular weight of 15,000, and did not have an inhibitory effect on plaque size at any concentration. This would be expected on the basis of the proposed theory.

There appear to be several basic differences in the mechanisms whereby polyanions increase the plaque size of some strains of poliovirus and of M-Mengo. Firstly, high molecular weight dextran sulfates which enhance plaque formation by type 1 polio variants (Takemoto and Liebhaver, 1962b; Takemoto and Kirschstein, 1964) strongly inhibit that of M-Mengo. Secondly, enhancement of M-Mengo plaque size by low molecular weight

polyanions is not brought about by an increased production of virus or by an earlier release of virus from the cells, as appears to be the case with poliovirus (Takemoto and Liebhaver, 1962b). The present results do not offer an alternative, but they indicate that the mechanism of plaque enhancement may be different in the two virus systems.

Heparin exerts no microscopically detectable toxic action on human amnion cells, even at a concentration of 10 mg/ml, and these cells failed to take up any detectable amount of heparin as measured by metachromatic staining with toluidine blue (Vaheri, 1964). Anionic polymers, including heparin, do not inhibit the attachment of HeLa cells to glass in the absence of serum, but cause cells to clump in serum-containing medium (Nordling et al., 1965). Heparin inhibits hyaluronidase, and a positive correlation between the clumping effect and a hyaluronidase-inhibiting activity of a series of anionic polymers was found (Nordling et al., 1965). Polyphlorethin phosphate and polyphloroglucinol phosphate, like heparin, enhanced the size of M-Mengo plaques. Both these compounds strongly inhibit hyaluronidase. In addition, heparin has been shown to elicit the protrusion of mandrake-like extensions from sheep erythrocytes (Litschel and Tomcsik, 1965). These observations suggest the possibility that heparin and other low molecular weight polyanions may produce some morphological alteration of the L cell surface and that this, in turn, may result in a faster cell-to-cell transfer of Mengo virus.

CHAPTER 6

Properties of Other Mengo Variants Isolated from S- and M-Mengo Pools

Introduction

During the studies of the effects of polyions on the sizes of the plaques produced by the three Mengo variants (Chapter 5), it became apparent that at least two of the variants (S and M) were not as homogeneous as was originally thought. In Chapter 3, the isolation and some properties of a fourth variant, SL-Mengo, were briefly described. In the intervening time since the detection of this variant, five others have been isolated--three from pools of S-Mengo, and two from pools of M-Mengo. The present chapter describes the isolation and some properties of all six new variants, and considers their relationship to the three original variants, S-, M-, and L-Mengo.

Materials and Methods

Overlays

The overlays used to detect the new variants have all been described in Chapter 5. Basically, regular agar overlay was used throughout, with or without the addition of polyions, or with agarose replacing the Noble agar.

Cloning procedure, and preparation of pools

As far as possible, only plaques well separated from neighboring plaques were selected for cloning. The whole area of a plaque (or part of it, depending on the size),

together with the overlying agar, was removed by means of a sterile Pasteur pipette, and mixed with 2-3 ml of virus diluent. Dilutions of this suspension were replated on L cells, and the plaques produced under several different overlays were checked for homogeneity. If not homogeneous, i.e. if the progeny virus did not breed true, the cloning procedure was repeated with a fresh plaque. If all the plaques were of a uniform type, the virus suspension was used to infect a monolayer of L cells in a milk dilution bottle. Virus produced was again checked for homogeneity by plating on L cell monolayers, and was used to prepare a pool as described in Routine Materials and Methods.

Results

Isolation of new variants from S-Mengo pools

Since the original isolation (Ellem and Colter, 1961), pools of S-Mengo have given rise to extremely small plaques when plated in L cells under regular agar overlay. On rare occasions, however, single large, L-Mengo-like plaques, which, on cloning, bred true, were obtained among the minute ones. The occasional appearance of these large plaques was attributed to a tendency of S-Mengo to mutate, with low frequency, to L-Mengo, and the matter was not investigated further. At this stage (before the studies reported in Chapters 4 and 5 were done), there was no reason to believe that any variants other than S, M, and L existed in pools of the three variants.

I have been thinking of you very much lately, and wondering how you are getting on. I hope you are well and happy. I have been very busy lately, but I have managed to find some time to write to you. I have been thinking of you very much lately, and wondering how you are getting on. I hope you are well and happy. I have been very busy lately, but I have managed to find some time to write to you.

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Isolation of SL-Mengo. As described in Chapter 5, the replacement of agar in regular agar overlay by agarose resulted in the enlargement of S-Mengo plaques to an average 2.0 mm diameter. In addition to these medium-sized plaques, however, a small percentage (0.1-1%) of larger (5-7 mm diameter) plaques were obtained. Through five successive clonings, virus from these plaques bred true, producing large, diffuse plaques under agarose overlay, but minute plaques, almost indistinguishable from those of S-Mengo, under agar overlay. Since the morphology of plaques produced by this new variant under agarose overlay resembled that of L-Mengo plaques under regular agar overlay, it was named SL-Mengo, and virus from a single plaque of the progeny of the fifth cloning was used to prepare a pool. Further repeated clonings of plaques produced from this pool failed to indicate any reversion to S-Mengo.

Isolation of SN-, SX-, and SH-Mengo. These three variants were isolated from the same pool of SL-Mengo, during a series of experiments to investigate the effects of dextran sulfates of low molecular weight on the sizes of plaques produced by SL-Mengo under agar overlay. Although SL-Mengo appeared to be homogeneous under agarose overlay, under agar overlay containing 1-2 mg/ml of dextran sulfate type 15S (Sigma; molecular weight 15-20,000), some heterogeneity was noted. Repeated clonings of numerous plaques resulted in the isolation of three distinct variants, which were designated SN, SX, and SH. The letters "N, X, and H" are not descriptive of the sizes of plaques produced by these variants, and have no real significance.

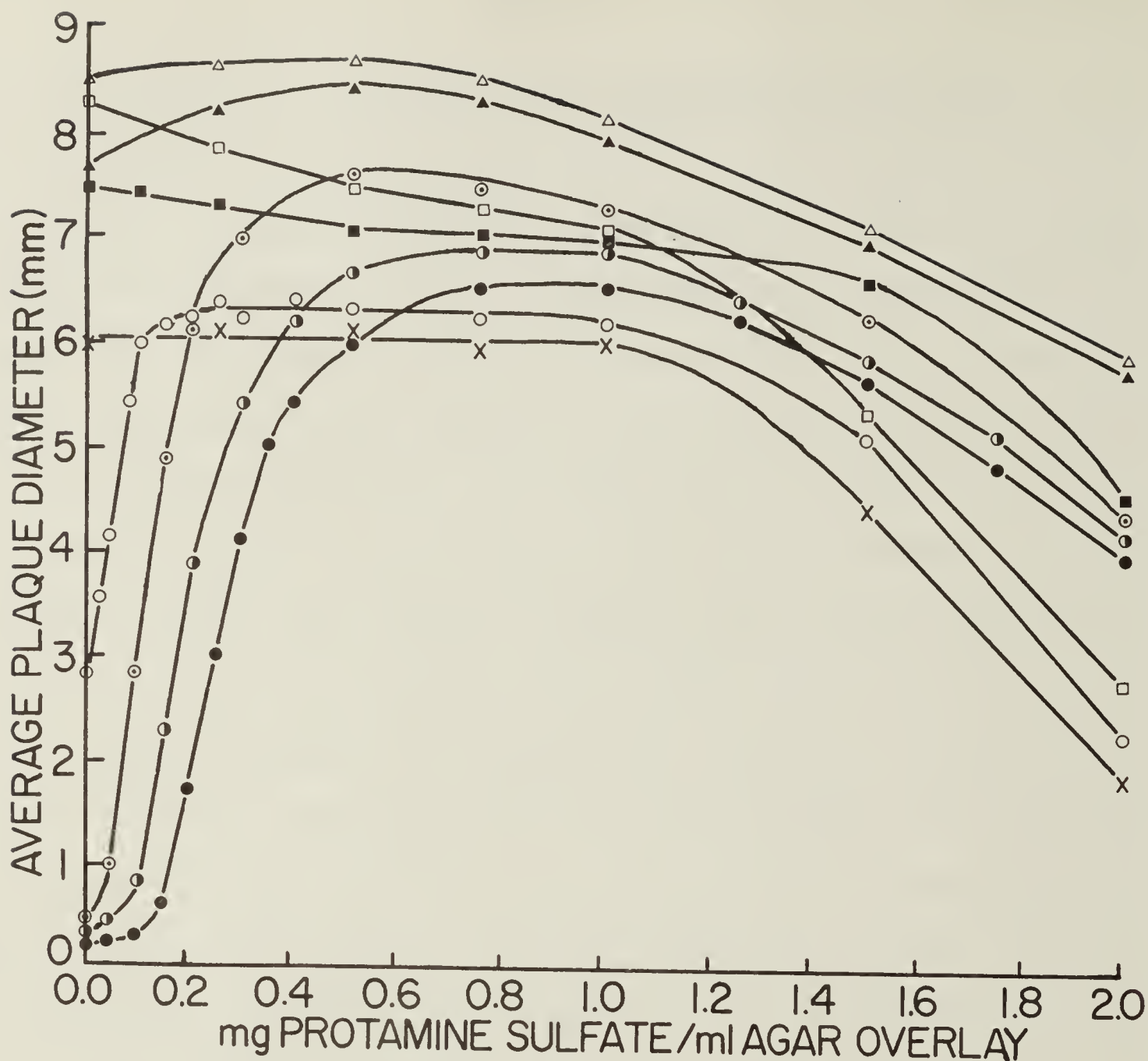


Figure 6.1. The effect of the concentration of protamine sulfate in the agar overlay on the size of the plaques produced by all nine Mengo variants in L cell monolayers. ● = S-Mengo; ● = SL-Mengo; ⊙ = SN-Mengo; ○ = M-Mengo; ○ = L-Mengo; ■ = MB-Mengo; ▲ = SX-Mengo; □ = MA-Mengo; △ = SH-Mengo.

Isolation of MA- and MB-Mengo

Although pools of M-Mengo almost invariably gave medium-sized, ragged plaques when plated under regular agar overlay, an occasional large, diffuse plaque was also obtained. As with the S variant, this was originally attributed to a tendency of M-Mengo to mutate, with low frequency, to L-Mengo. The experiments concerning the effects of dextran sulfate in the agar overlay on the plaque sizes of M-Mengo (see Figure 5.4) indicated, however, that this conclusion would have to be revised.

The addition of dextran sulfate (Pharmacia; molecular weight 500,000) to regular agar overlay resulted in the sizes of plaques produced by M-Mengo being reduced to about 0.3 mm in diameter (Figure 5.4). In addition, however, an occasional plaque was obtained which was not inhibited to such an extent. Several of these plaques were picked, and after repeated cloning, two similar, though distinct variants were isolated, which produced large plaques under regular agar overlay. Pools of these were prepared, and were designated MA- and MB-Mengo.

Effect of protamine sulfate on the plaque sizes of the variants

The fact that the six new variants were isolated under overlays which differed from regular agar overlay with respect to the amount of polyanionic or polycationic material they contained, suggested that they differed in their sensitivities to polyions. An extension of the studies presented in Figure 4.1 was therefore undertaken, to examine the effects of adding

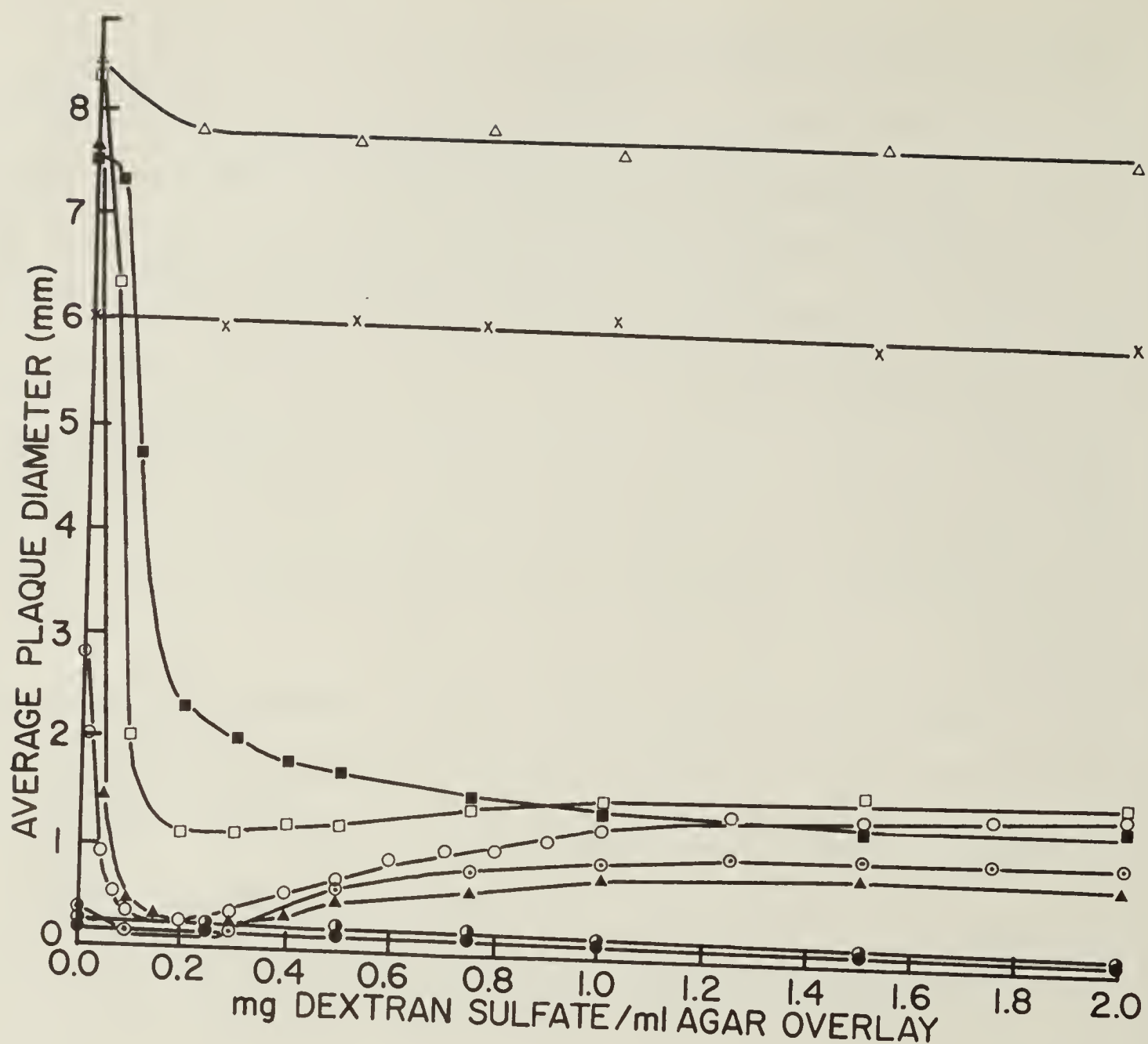


Figure 6.2. The effect of the concentration of dextran sulfate (Pharmacia) in the agar overlay on the size of the plaques produced by all nine Mengo variants in L cell monolayers. ● = S-Mengo; ◐ = SL-Mengo; ◑ = SN-Mengo; ◒ = M-Mengo; x = L-Mengo; ■ = MB-Mengo; ▲ = SX-Mengo; ◻ = MA-Mengo; Δ = SH-Mengo.

protamine sulfate to regular agar overlay on the sizes of plaques produced by all nine variants. The results of this further study are summarized in Figure 6.1. It should be noted that no reduction in plaque numbers was found over the range 0-1 mg protamine sulfate/ml of overlay, although above this concentration considerable reduction in plaque numbers occurred.

The most interesting feature of this figure are the curves of SN- and SL-Mengo at low concentrations of protamine, which appear to trisect the area between the M- and S-Mengo curves. In fact, the four curves (M, SN, SL, and S) are almost parallel, with an interval equivalent to about 80 μ g protamine/ml between each one.

Effect of dextran sulfate on the plaque sizes of the variants

The same procedure was carried out with dextran sulfate 500 (Pharmacia), extending the data provided in Figure 5.4 to include all nine variants. A summary of the results is shown in Figure 6.2. SH-Mengo was found to be almost insensitive to this polyanion, although all the other variants, with the exception of L-Mengo, were strongly inhibited.

A comparative study of the effect of low molecular weight dextran sulfate on the plaque sizes of the variants other than M-Mengo has not been carried out, but data have been obtained for S- and SL-Mengo with type 15S material (Sigma). Table 6.1 summarizes the results of three experiments.

Table 6.1

Effect of Type 15S* Dextran Sulfate Concentration in Regular Agar Overlay on S- and SL-Mengo Plaque Sizes in L Cells

Dextran sulfate concentration in regular agar overlay (μ g/ml)	Average plaque diameter after 72 hours incubation (mm)	
	SL-Mengo	S-Mengo
0	0.2	0.2
250	0.4	0.6
500	0.8	1.1
750	1.1	1.4
1000	1.4	1.9
1500	1.8	2.0
2000	1.9	2.1

* Sigma; molecular weight 15-20,000.

Sulfopolyglucin appeared to have a similar effect on SL-Mengo plaque size as did the type 15S material. Of the higher molecular weight materials tested (Sigma types 200S and 5-40S; Pharmacia 500) and heparin, none had any appreciable effect on the size of plaques produced by SL-Mengo under agar overlay.

Virulence of the Mengo variants in mice

In view of the marked difference in the virulence of the original variants to mice when administered by the intraperitoneal route, it was of considerable interest to determine the intraperitoneal LD₅₀'s of the six new variants. The same

procedure was used as is described in Chapter 3. Groups of five 14-16 g male mice were injected intraperitoneally with tenfold dilutions of virus, and the animals were observed, and all deaths recorded, for 12 days thereafter. The method of Reed and Muench (1938) was used to calculate the LD₅₀'s, which are presented in Table 6.2. The variants are listed in order of increasing plaque size produced under regular agar overlay. It is evident that there is no simple relationship between virulence and plaque size.

Table 6.2

Virulence of the Nine Mengo Variants in Mice
after Intraperitoneal Injection

Variant	Intraperitoneal LD ₅₀ (PFU)	Average plaque diameter (mm) *
S	> 10,000,000	0.2
SL	10	0.3
SN	50-500	0.4
M	10-50,000	2.8
L	1	6.0
SX	4-40	7.5
MB	16	7.7
MA	320	8.3
SH	1	8.6

*After 72 hours incubation under regular agar overlay.

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Table 1. Summary of data for the first 10 years of the study.		
Year	Number of subjects	Number of events
1980	100	10
1981	100	12
1982	100	15
1983	100	18
1984	100	20
1985	100	22
1986	100	25
1987	100	28
1988	100	30
1989	100	32
1990	100	35

The data for the first 10 years of the study are summarized in Table 1. The number of subjects in the study was 100 in each year. The number of events was 10 in 1980, 12 in 1981, 15 in 1982, 18 in 1983, 20 in 1984, 22 in 1985, 25 in 1986, 28 in 1987, 30 in 1988, and 32 in 1989. The number of events in 1990 was 35.

Discussion

Of the nine Mengo variants described in this chapter (the three original and six new ones), the plaque sizes of seven are inhibited by polyanions (Figure 6.2). Although no complete correlation can be found between this factor and the apparent virulence of the variants after intraperitoneal injection into mice (Table 6.2), it should be noted that the most virulent of them--L- and SH-Mengo--are the only ones capable of producing, under agar overlay, plaques which are almost completely resistant to inhibition by sulfated agar components or by dextran sulfate (Figure 6.2). Hence, the suggestion of Agol and Chumakova (1963), that the variant of a virus which is least sensitive to the action of polyanions should be the most pathogenic, is partly borne out by the present study.

It seems reasonably clear that the large, diffuse plaques occasionally encountered during the growth of S- and M-Mengo under regular agar overlay were plaques of SX-, SH-, MA-, or MB-Mengo rather than L-Mengo, as was originally supposed. L-Mengo was never isolated from pools of S- or M-Mengo during the entire period of the search for new variants (about two years), although this is not complete proof that the S and M variants cannot mutate to L.

The marked heterogeneity in the size of plaques produced by L-Mengo appears to be an inherent property of this variant, since all attempts to isolate other plaque variants from pools of L-Mengo have so far failed. SH-Mengo, although similar to L-Mengo in that its plaque size is little affected

Table 6.3

Effect of Dextran Sulfate and Protamine on the Plaque Sizes of the Mengo Variants

Overlay	Mengo variant									
	S	SL	SN	M	L	MB	SX	MA	SH	
Noble agar (1%)	.	.	.	•	•	•	•	•	•	
Noble agar + 150 µg/ml protamine sulfate	.	•	•	•	•	•	•	•	•	
Noble agar + 150 µg/ml dextran sulfate 500	•	•	.	.	•	

by either dextran sulfate or protamine, can be readily distinguished from the latter by the larger, more uniformly-sized plaques it produces under regular agar overlay.

Table 6.3 illustrates how the nine variants can be distinguished from each other by use of only three overlays--regular agar overlay itself, or regular agar overlay containing 100-150 $\mu\text{g/ml}$ of either dextran sulfate 500 (Pharmacia) or of protamine sulfate. Differentiation of MB-, MA-, SX-, and SH-Mengo can be obtained under overlay containing dextran sulfate, and S-, SL-, and SN-Mengo can be separated under overlay containing protamine sulfate.

It must be noted that although the relative sensitivities of the variants to inhibition or enhancement of plaque size by polyions did not alter, the absolute diameters of plaques produced by individual variants frequently varied considerably from experiment to experiment. The reason for this is probably that several factors other than the presence or absence of polyions in the overlay are involved in the determination of plaque size. Table 6.4 illustrates how differences in the bicarbonate concentration in the overlay, affecting the pH of incubation, can also affect the sizes of plaques produced by the three original variants. Slight variations in the CO_2 content of the air in the incubators could therefore cause some of the variation in plaque size. Another cause could be slight fluctuations in the temperature of incubation--the marked effect of temperature on the growth of viruses has been well documented (see e.g. Ruiz-Gomez and Isaacs, 1963a). It has also been found that different batches of the same type

Table 6.4

Effect of pH of Overlay on Mengo Plaque Sizes

pH of overlay	Average plaque diameter (mm)		
	L-Mengo	M-Mengo	S-Mengo
6.8	3.1	0.1	<0.1
7.2	5.3	0.6	0.2
7.6	7.1	4.3	0.3

Plaques were developed for 72 hours in L cell monolayers incubated at 37° in an atmosphere of 5% CO₂ in air, under regular agar overlay modified to contain sufficient bicarbonate to maintain the pH at the required value. The pH's were measured on fluid from control monolayers incubated under the same conditions but without agar in the overlay medium.

of agar routinely used (Noble agar) varied slightly in their content of saline-extractable material, and this is undoubtedly a contributing factor.

It would be extremely difficult to control completely even these variables, and any attempt to do so, apart from normal routine checks, was not considered worth the effort. Instead, multiple determinations of plaque sizes were carried out, and every point in Figures 6.1 and 6.2 is the averaged value of the results from two to eight separate experiments (the number of experiments carried out depending on the degree of reproducibility obtained).

In view of this variation, and since some of the variants are very closely related in their sensitivities to polyions, it is not possible to identify positively an unknown

variant by comparing the size of plaques it produces under a variety of different overlays with the values given in Figures 6.1 and 6.2. Comparison with established variants is essential, and the following scheme has been found to be useful in identifying an unknown variant. By utilising the three overlays described in Table 6.3, and by including several established variants with the unknown, a tentative identification of the latter can be made. This can then be confirmed by comparison of the unknown with the corresponding standard when both are plated under a more complete range of overlays. If the unknown corresponds to none of the standards, it can be considered to be a new variant.

DISCUSSION AND CONCLUSIONS

The most frustrating part of the present work has been the failure to give a satisfactory explanation for the marked virulence of L-Mengo when injected intraperitoneally into mice. By almost all criteria examined, L-Mengo should have been the least virulent of the three variants. It has the lowest burst size (Table 1.1), and attaches least readily to L cells and to tissue homogenates (Tables 2.1 and 3.2); properties which would not be expected in the most lethal variant. Moreover, its sensitivity to exogenous interferon, and the fact that it apparently stimulates the production of more interferon in vivo (Chapter 3) than the other variants, is indicative of an avirulent rather than a virulent character (Wagner, 1965).

Two other distinguishing properties of L-Mengo--its relative insensitivity to inhibition by agar factor (Chapter 4) and its relative stability at acid pH (Chapter 2), do not appear to offer an explanation. For a time, it was considered that since L-Mengo--L cell interaction was the least sensitive to inhibition by agar factor, this might imply that L-Mengo was less inhibited by naturally occurring polyanions in vivo than the other two variants, and would therefore be able to spread more easily in the animal body. This theory now seems less likely, however, in view of the rather unexpected discovery that, of the three variants, the interaction of L-Mengo with L cells is by far the most strongly

inhibited by heparin--a naturally occurring, polyanionic tissue constituent (Table 5.5). It is difficult also to find a connection between the relative acid stability of L-Mengo and its neurovirulence, since the pH of the animal body will not vary appreciably from 7.4, at which pH all three variants are quite stable.

One factor which has not been taken into account is the effect of temperature on the course of viral infection. All the studies presented in this thesis involving viral growth have been carried out at an average temperature of 37°. Lwoff (1962) however, has found that when viral development is followed in single growth cycles as a function of temperature, the incubation temperature affects: (i) the length of the eclipse period, (ii) the rate of viral development, and (iii) the final virus yield. A relation between the ability to develop at high temperature and neurovirulence has been established for poliovirus types 1 and 2 (Lwoff et al., 1959; Lwoff, 1961; Sabin, 1961), EMC virus (Pérol-Vauchez et al., 1961), foot and mouth disease virus (Prunet, 1964), and vaccinia virus (Kirn and Braunwald, 1964; Kirn, 1965). The internal (rectal) temperature of mice maintained at 25° has been found to vary from 37-38°, and mice kept at 4° and 36° had rectal temperatures of 36-37° and 39-40° respectively (Walker and Boring, 1958). In keeping with these observations, Pérol-Vauchez et al. (1961) noted average rectal temperatures of 36.5°, 38°, and 39.5° in mice maintained respectively at 4°, 20°, and 35°. It has also been found that Cocksackie B1 virus, which in normal mice produces an initial

viremia followed by significant viral multiplication only in the pancreas, multiplied to fairly high levels and produced marked damage in several tissues of mice kept at 4° (Walker and Boring, 1958; Walker, 1963). Interpretation of the results of these in vivo studies with animals kept at 4° are complicated by the fact that stress, or other physiological changes due to cold, may contribute to the reaction: the studies of Lwoff and others referred to above, however, with in vitro systems, cannot be criticized on these grounds.

It is possible, then, that an investigation of the growth rates of the three variants at temperatures above and below 37° may reveal that the optimum temperature for L-Mengo growth is above 37°, and that of the other two variants is at 37° or below. If this is in fact the case, the in vitro determinations of the growth rates of the three variants at 37° (Chapter 1) will not have given a true picture of their growth rates in mice in vivo, at a possibly higher temperature. At even 38°, the growth of S- and M-Mengo may be considerably lower, and that of L-Mengo considerably higher, than at 37°. This hypothesis might therefore explain the longer eclipse period and the lower burst size of L-Mengo as compared with M-Mengo (Figures 1.2 and 1.3; cf. Lwoff, 1962). The reason that S-Mengo (Figure 1.1) has an eclipse period of the same duration as does L-Mengo may be that while 37° is a suboptimal temperature for the latter, it is a supraoptimal one for the former, and in both cases this would lead to a reduced efficiency of viral multiplication at this temperature.

The following table shows the results of the experiments conducted on the effect of the various factors on the rate of the reaction. The results are given in the form of a table, the columns of which are headed by the names of the factors, and the rows by the names of the experiments. The numbers in the cells of the table represent the rate of the reaction, as determined by the method described in the text.

Experiment	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
1	0.1	0.2	0.3	0.4	0.5
2	0.2	0.3	0.4	0.5	0.6
3	0.3	0.4	0.5	0.6	0.7
4	0.4	0.5	0.6	0.7	0.8
5	0.5	0.6	0.7	0.8	0.9
6	0.6	0.7	0.8	0.9	1.0
7	0.7	0.8	0.9	1.0	1.1
8	0.8	0.9	1.0	1.1	1.2
9	0.9	1.0	1.1	1.2	1.3
10	1.0	1.1	1.2	1.3	1.4

The results of the experiments show that the rate of the reaction increases with the increase of the concentration of the reactants. The rate of the reaction is also affected by the temperature, the presence of a catalyst, and the surface area of the reactants. The rate of the reaction is highest when all the factors are at their maximum values, and lowest when all the factors are at their minimum values.

In brief, then, the proposed hypothesis is that the optimal temperature for L-Mengo growth is above 37° , that of M-Mengo is around 37° , and that of S-Mengo is below this temperature. This hypothesis would also provide an explanation for the relative intraperitoneal LD₅₀'s of the three variants (Table 3.1) in terms of the relationship between neurovirulence and optimal growth temperature established by Lwoff and others, and temperature would be the "barrier system" in mice which prevents the access of M- and S-Mengo to the nervous system (see Chapter 3, Discussion).

The proposed theory does not fit in with the general rule observed by Ruiz-Gomez and Isaacs that virulent viruses with high optimum temperatures have a low sensitivity to interferon in comparison with less virulent strains. Nevertheless, exceptions to this rule have been found. Kumba virus, with an optimal temperature of 35° , and with a relatively high sensitivity to the antiviral action of interferon, is highly virulent in chick embryos. Yellow fever (17D strain), with an optimal temperature of 37° and a low sensitivity to the antiviral action of interferon, shows a very low virulence in chick embryos (Ruiz-Gomez and Isaacs, 1963a). A possible complicating factor in these studies is the varying total:infectious particle ratio for different viruses. It is not known whether all the particles in a virus population, or simply the infectious or noninfectious fraction, contribute to the formation of interferon. If only the noninfectious particles contribute, and if, as indicated in Chapter 1, L-Mengo has the largest total:infectious particle ratio, this

might explain why, in the present work, the L variant appears to be the most efficient producer of interferon.

It is realized, however, that to advance this hypothesis, without any solid facts to support it, is akin to skating on rather thin ice. Assessment of this discussion may therefore have to await the results of growth studies of the variants at temperatures above and below 37⁰, determinations of their total:infectious particle ratios, and more complete studies of their sensitivity to, and efficiency in producing, interferon. Although preliminary experiments on the production of, and sensitivity to, interferon by the three Mengo variants have been reported in Chapter 3, several considerations should be kept in mind in evaluating these results. It should be noted that although the actual substance, or substances, responsible for the inhibition of viral growth have been referred to as interferon, the preparations used were not characterized fully as such. A full characterization of any material suspected of containing interferon entails demonstration that it possesses chemical and physical properties consistent with the present views on the nature of interferon: namely, stability at low and high pH, ability to inhibit heterologous viruses, species specificity, non-sedimentability at 100,000 g, inactivation by proteolytic enzymes, and insensitivity to nucleases and lipases (see Ho, 1964). Little attempt has been made so far to verify that the interferon preparations described in the present work actually meet all these requirements. Nevertheless, they were resistant to low pH, were not specific for the infecting virus, and were present only in infected cell cultures

or animals. The possibility that viral interference was mediated by non-infectious virus rather than interferon produced by infected cells is unlikely since in the in vivo experiments, the capacity of a tissue homogenate to interfere was not necessarily related to its virus content. It is very likely, therefore, that the material described in Chapter 3 as interferon is in fact an interferon, although it has not been fully characterized as such. This work then, still remains to be done.

It has been shown in Chapters 4 and 5 that the differences in the size of the plaques produced in L cell monolayers by the three Mengo variants can be satisfactorily explained on the basis of their differing sensitivity to inhibition by sulfated polysaccharides in agar. Under agar overlay in which these inhibitors have been inactivated by the addition of protamine, S- and M-Mengo produce slightly larger plaques than L-Mengo (Figure 4.1). This is consistent with the larger burst sizes of the S and M variants compared to L-Mengo (Table 1.1) and to the fact that they attach more readily to L cells (Table 2.1).

The studies presented in Chapter 4 clearly indicate that agar factor does not interfere with Mengo virus--L cell interaction by blocking cellular receptor sites, and Table 4.4 presents results to show that agar inhibitor is not taken up by the cells at all. However, in a very recent report concerning the inhibition of the growth of group A arboviruses in chick fibroblasts by an agar extract, Colón et al. (1965) found that although the agar inhibitor appeared to bind

directly to the virus, it also appeared to inhibit viral growth by combining with the cell surface. These workers suggested that two polysaccharides were present in the agar extract: one interacting with the virus particles, and another with the host cells. They found that the agar extract could be separated into two fractions by chromatography, although for some unexplained reason no inhibitory activity was demonstrated with either fraction. The existence of two such inhibitory polysaccharides in agar extract seems to be unlikely, however, and need not be postulated in order to explain their results.

Takemoto and Liebhaver (1961) have described the isolation of two plaque variants of EMC virus. Wild-type virus (r^+) produced minute plaques (1 mm at 4 days) in L cell monolayers, but an occasional mutant (r) was obtained which produced large plaques (8-10 mm at 4 days). The small plaque variant was shown to be inhibited by sulfated agar polysaccharides to which the large plaque variant was resistant. Craighead (1965a) has studied the pathogenicity of these two EMC variants for mice, and his results imply that the large plaque variant is virulent and that the small plaque one is avirulent. Any virulence displayed by the small plaque former when injected intraperitoneally appeared to be due to mutation to the r variant or some other large plaque former. This is of interest in view of the suggestion that S-Mengo is completely non-neurovirulent when injected intraperitoneally, and that any virulence attributed to the S variant is in fact due to its mutation to SL-Mengo (Chapter 3). The difference between

The first thing I noticed when I stepped out of the car was the
familiar smell of the city. It was a mix of old and new, of
history and progress. The air was thick with the scent of
coffee from the nearby cafes and the faint aroma of
flowers from the gardens. I took a deep breath and felt
myself being pulled into the heart of the city. The streets
were wide and clean, with a few cars and a few
pedestrians. The buildings were tall and modern, with
glass facades that reflected the sunlight. I walked
down the street, feeling a sense of wonder and
excitement. The city was beautiful, and I was
glad to be here. I had heard so much about it, and
now I was finally seeing it with my own eyes. The
city was a mix of old and new, of history and
progress. It was a place where the past and the
future met, and I was glad to be a part of it.

the two virus systems is that whereas the virulent, large-plaque EMC variant is insensitive to inhibition by sulfated agar constituents, the virulent SL-Mengo is only slightly less sensitive to inhibition by these than S-Mengo (Figure 6.1). Therefore, although evidence for a possible relationship between virulence and sensitivity to agar inhibitors has been demonstrated for foot and mouth disease virus (Bengtsson et al., 1963) and poliovirus (Agol and Chumakova, 1963), as well as EMC virus (Craighead, 1965a), the existence of SL-Mengo indicates that there need not necessarily be a connection between these two markers.

The role of naturally-occurring polyanions as factors in the natural resistance to viral infection is uncertain. Indications have been obtained that heparin and the synthetic polyanion polyanethol sulfonate can protect rabbits against the pathogenic effects of Shope's fibroma virus (Higginbotham and Murillo, 1965). On the other hand, experimental observations can be quoted that argue against the suggestion that the presence of polyanions may alter the course of a viral infection in vivo. For example, it has been found that certain dextran sulfates which inhibit the multiplication of influenza virus in tissue culture and chick embryos, have no favorable effect on the course of influenza virus infection in mice (Styk et al., 1964). In the present studies also, the observations that the in vitro growth of the virulent SL-Mengo is inhibited by sulfated agar polysaccharides and that heparin strongly inhibits the attachment of the virulent L-Mengo to L cells (Table 5.5), do not suggest that polyanions

affect the multiplication of these variants in vivo.

The discussion in the preceding few pages has attempted a synthesis, with much speculation, of the work presented in this thesis. It has been suggested that although the properties of the virulent L variant resemble those of an avirulent virus, the in vitro conditions employed in this study may not have given a representative picture of the properties of this variant in vivo. It has been shown that the sizes of plaques produced by the three variants in L cells under agar overlay can be satisfactorily explained on the basis of their differing sensitivity to sulfated polysaccharides present in agar, and that the minor differences in the plaque sizes remaining after inactivation of these inhibitors may be explained by the relative burst sizes of the variants and their relative affinities for L cells. An attempt has been made to correlate virulence with sensitivity to polyanions, and although such a relation has been found with the three original variants, it has been concluded from studies of the six new variants (Chapter 6) that there need not be a connection between these two markers. Suggestions have also been made for future work, to test the validity of the hypotheses presented here.

Looking into the not too distant future, it is apparent that the present work has established a firm ground base from which to launch an assault on the third part of the long-term objective: to relate the biological markers to the biochemical and biophysical parameters of the Mengo variants. The present

existence of not three but nine variants, with the distinct possibility of still more to be isolated, will greatly complicate future studies. Nevertheless, the added insight gained into problems of logistics by studies of these new variants, will probably more than justify the considerably extra effort involved.

"But, Och! I backward cast my e'e
on prospects drear!

An' forward, tho' I canna see,
I guess an' fear."

Robert Burns: To a Mouse.

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the first of these is the fact that the number of cases of the disease is

very small.

The second is the fact that the disease is very rare.

The third is the fact that the disease is very dangerous.

The fourth is the fact that the disease is very difficult to treat.

The fifth is the fact that the disease is very common.

The sixth is the fact that the disease is very easy to catch.

The seventh is the fact that the disease is very fatal.

The eighth is the fact that the disease is very contagious.

The ninth is the fact that the disease is very infectious.

The tenth is the fact that the disease is very communicable.

The eleventh is the fact that the disease is very transmissible.

The twelfth is the fact that the disease is very contagious.

The thirteenth is the fact that the disease is very infectious.

The fourteenth is the fact that the disease is very communicable.

The fifteenth is the fact that the disease is very transmissible.

The sixteenth is the fact that the disease is very contagious.

The seventeenth is the fact that the disease is very infectious.

The eighteenth is the fact that the disease is very communicable.

The nineteenth is the fact that the disease is very transmissible.

The twentieth is the fact that the disease is very contagious.

The twenty-first is the fact that the disease is very infectious.

The twenty-second is the fact that the disease is very communicable.

The twenty-third is the fact that the disease is very transmissible.

The twenty-fourth is the fact that the disease is very contagious.

The twenty-fifth is the fact that the disease is very infectious.

The twenty-sixth is the fact that the disease is very communicable.

The twenty-seventh is the fact that the disease is very transmissible.

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The first part of the paper is devoted to a general discussion of the problem. It is shown that the problem is of great importance in the theory of the structure of the atom. The second part is devoted to a detailed discussion of the problem. It is shown that the problem is of great importance in the theory of the structure of the atom. The third part is devoted to a detailed discussion of the problem. It is shown that the problem is of great importance in the theory of the structure of the atom. The fourth part is devoted to a detailed discussion of the problem. It is shown that the problem is of great importance in the theory of the structure of the atom. The fifth part is devoted to a detailed discussion of the problem. It is shown that the problem is of great importance in the theory of the structure of the atom. The sixth part is devoted to a detailed discussion of the problem. It is shown that the problem is of great importance in the theory of the structure of the atom. The seventh part is devoted to a detailed discussion of the problem. It is shown that the problem is of great importance in the theory of the structure of the atom. The eighth part is devoted to a detailed discussion of the problem. It is shown that the problem is of great importance in the theory of the structure of the atom. The ninth part is devoted to a detailed discussion of the problem. It is shown that the problem is of great importance in the theory of the structure of the atom. The tenth part is devoted to a detailed discussion of the problem. It is shown that the problem is of great importance in the theory of the structure of the atom.



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